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Chang et al.

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(54) **PLANTS AND PROCESSES FOR OBTAINING THEM**

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(57) **ABSTRACT**

The invention relates to a transgenic or mutated plant having genomic material which alters the normal starch synthesis pathway within the plant. More specifically, the present invention relates to a plant having a genotype which creates new forms of starch in significant quantity. Particularly, the invention relates to grain having an embryo with a genotype heterozygous for two or more wild type genes (for example, Aa/Bb) and an endosperm having a genotype heterozygous for such genes (for example, AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB) and the starch produced therefrom.

11 Claims, 17 Drawing Sheets

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(52) **U.S. Cl.** **800/298; 800/263; 800/320.1**

(58) **Field of Search** 800/263, 266, 800/271, 320.1

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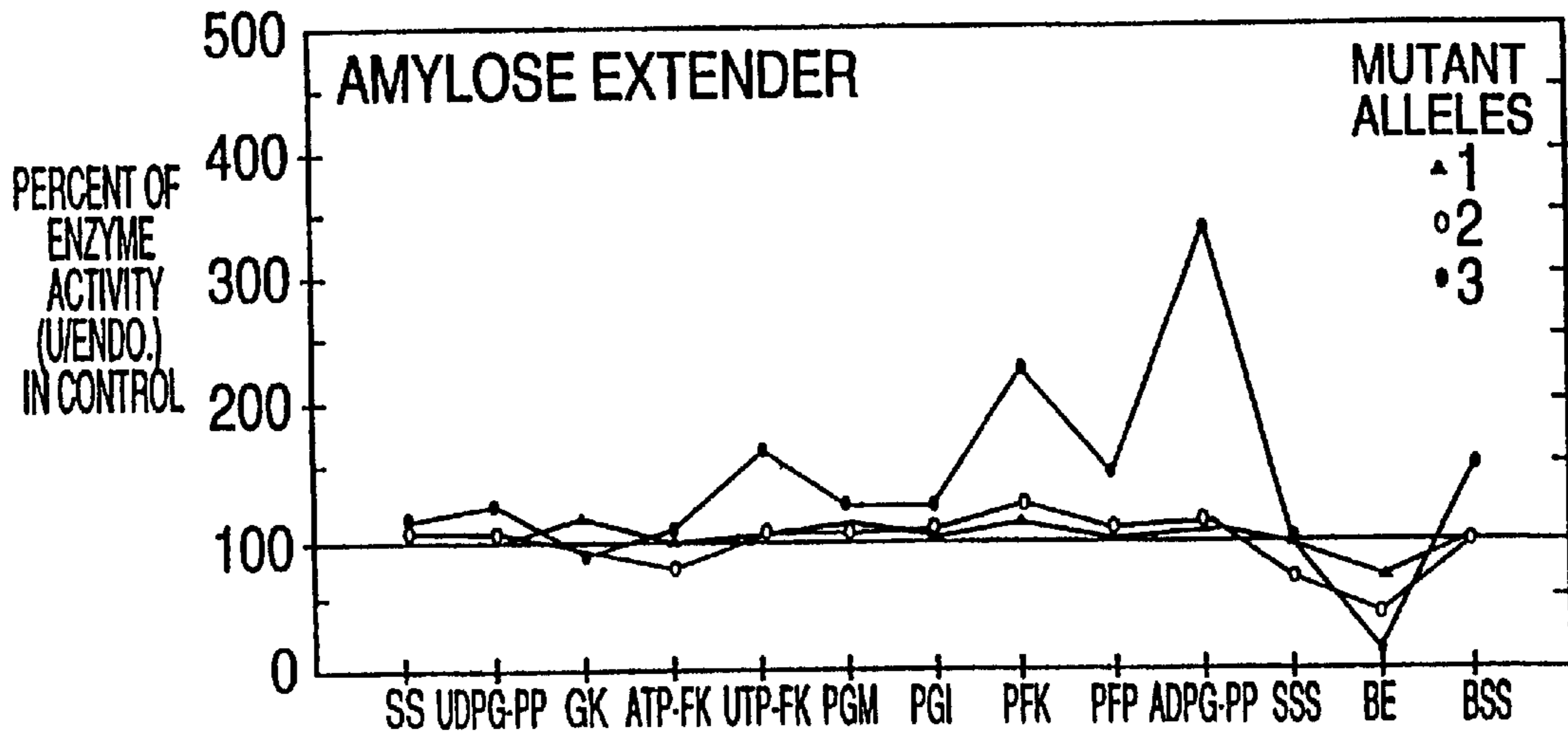
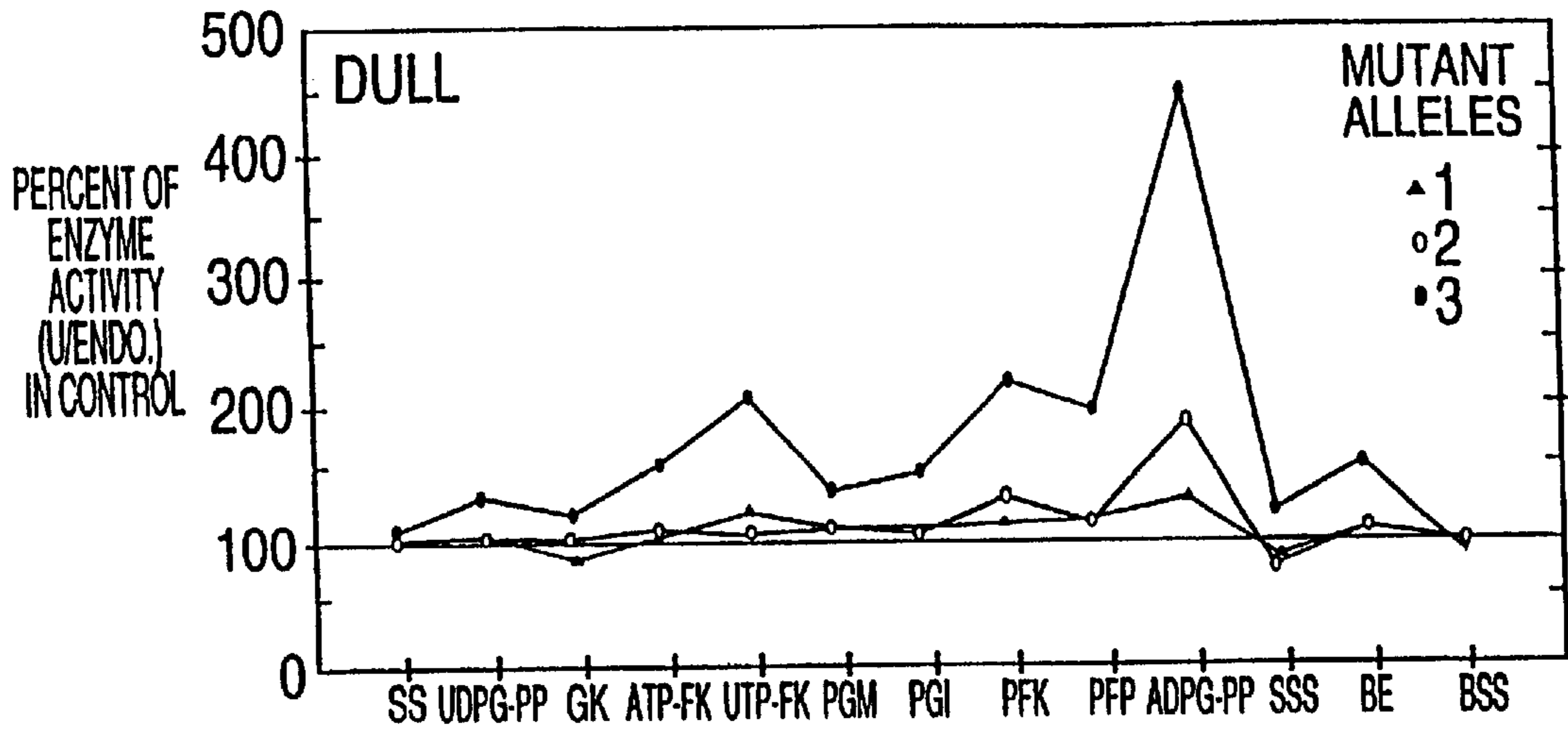


FIG. 1

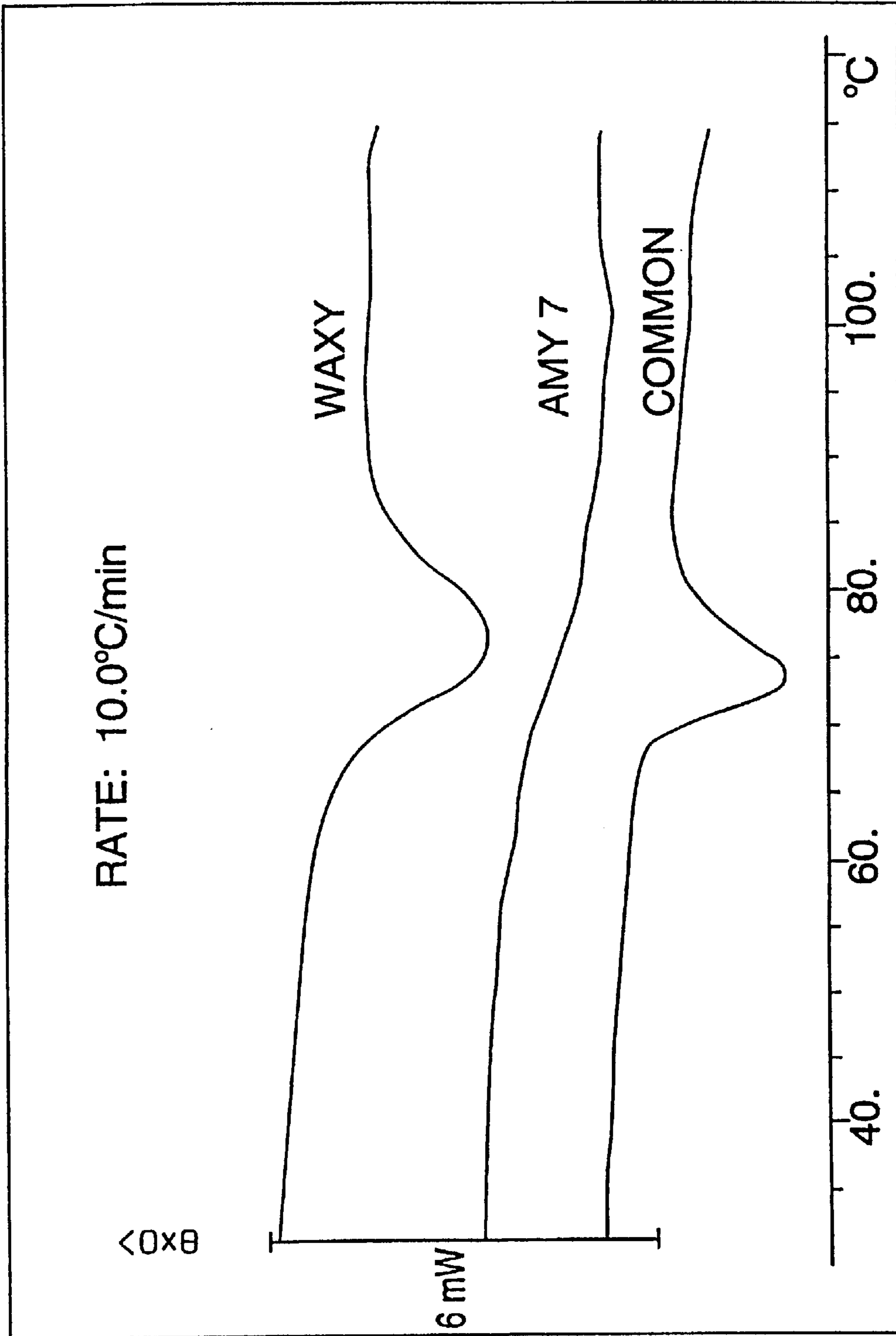


FIG. 2A

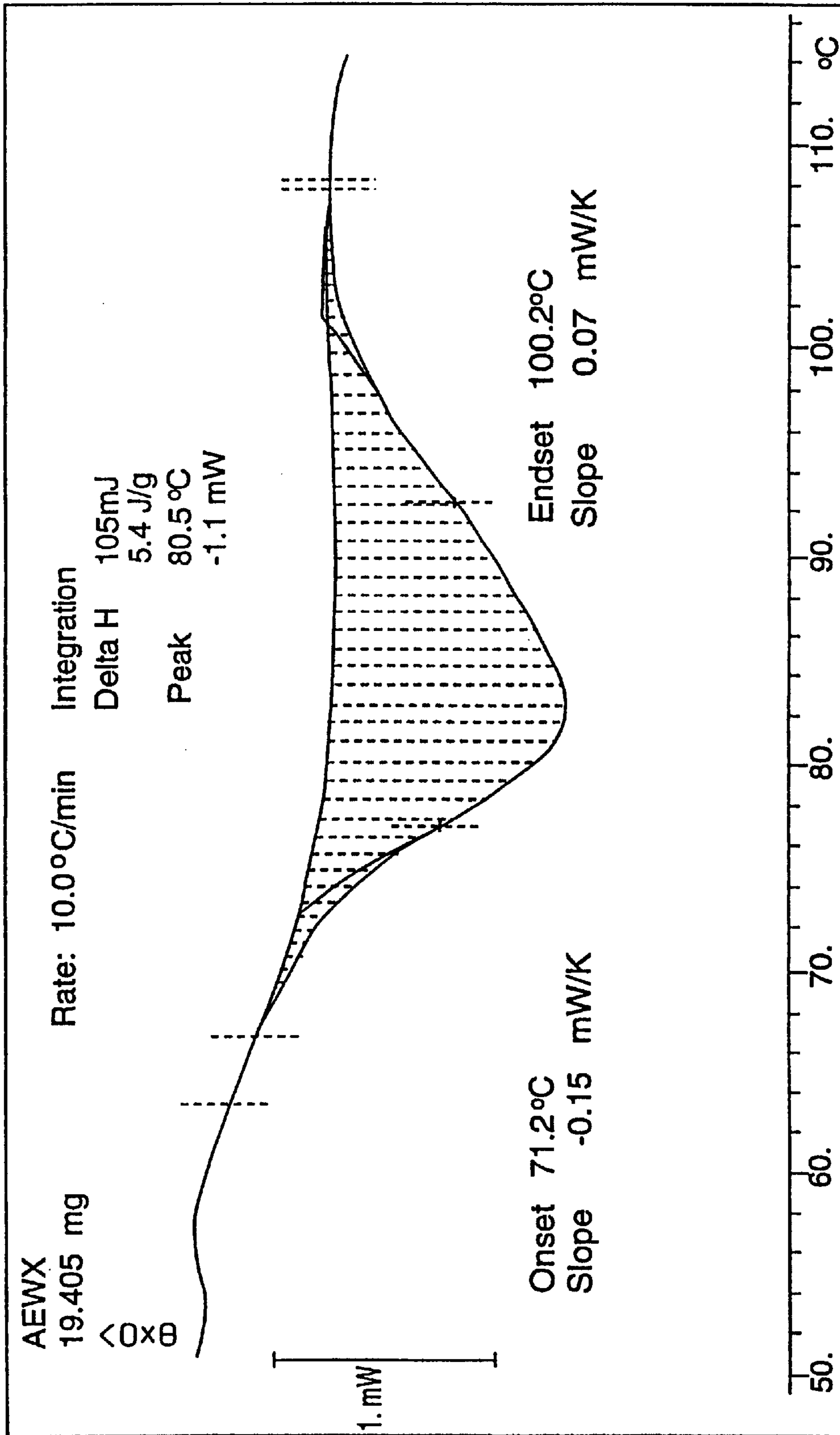


FIG. 2B

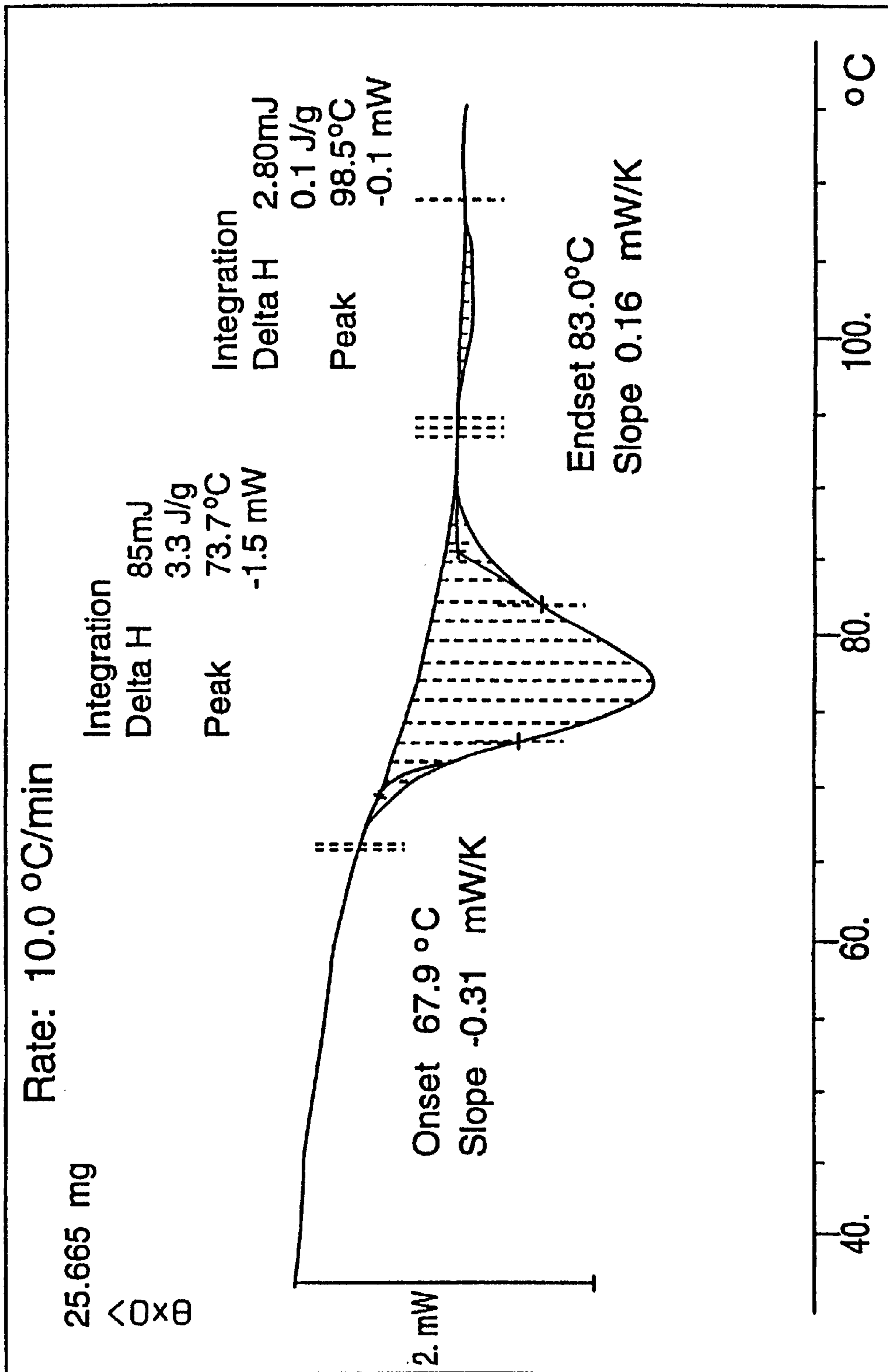


FIG. 2C

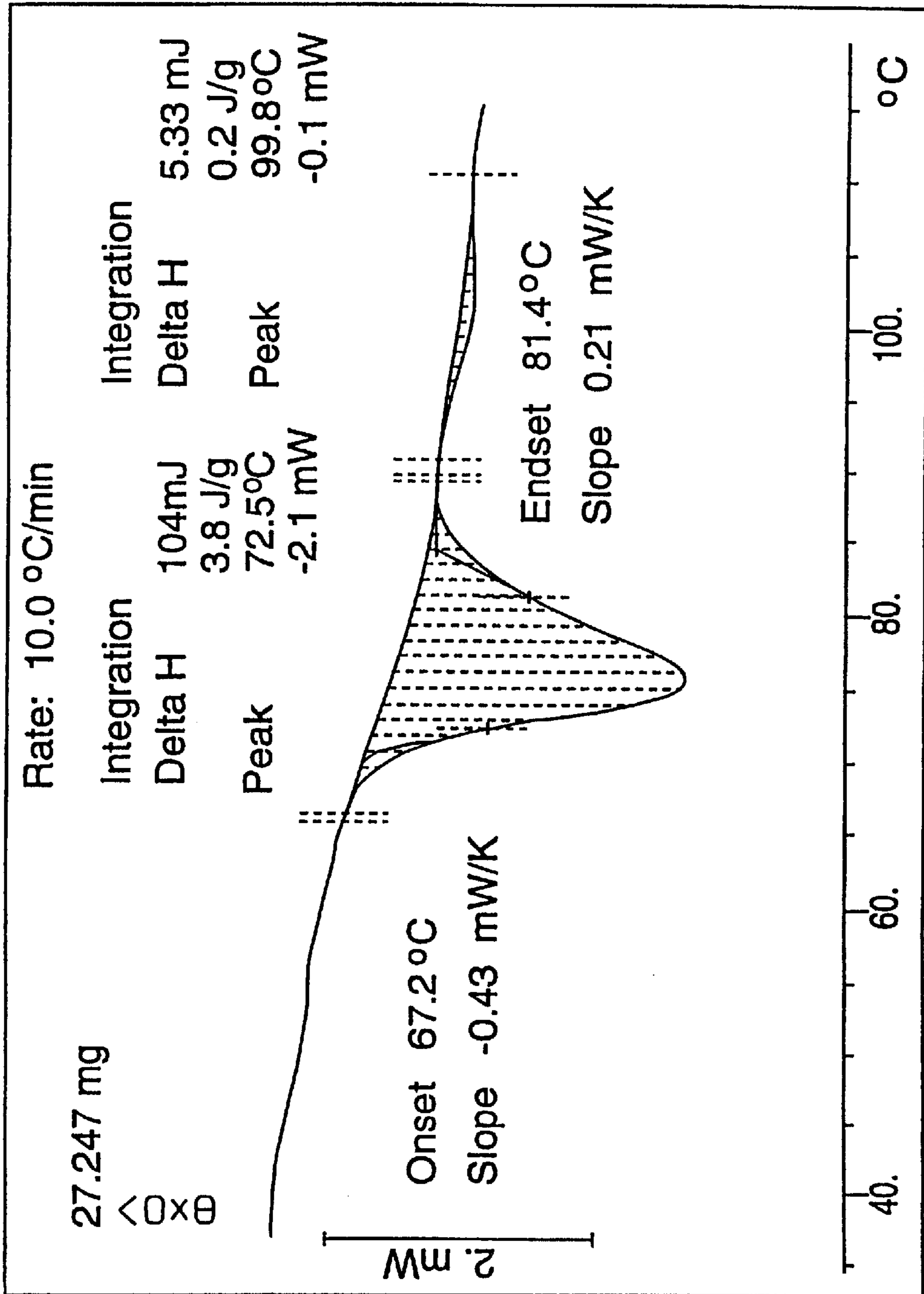


FIG. 2D

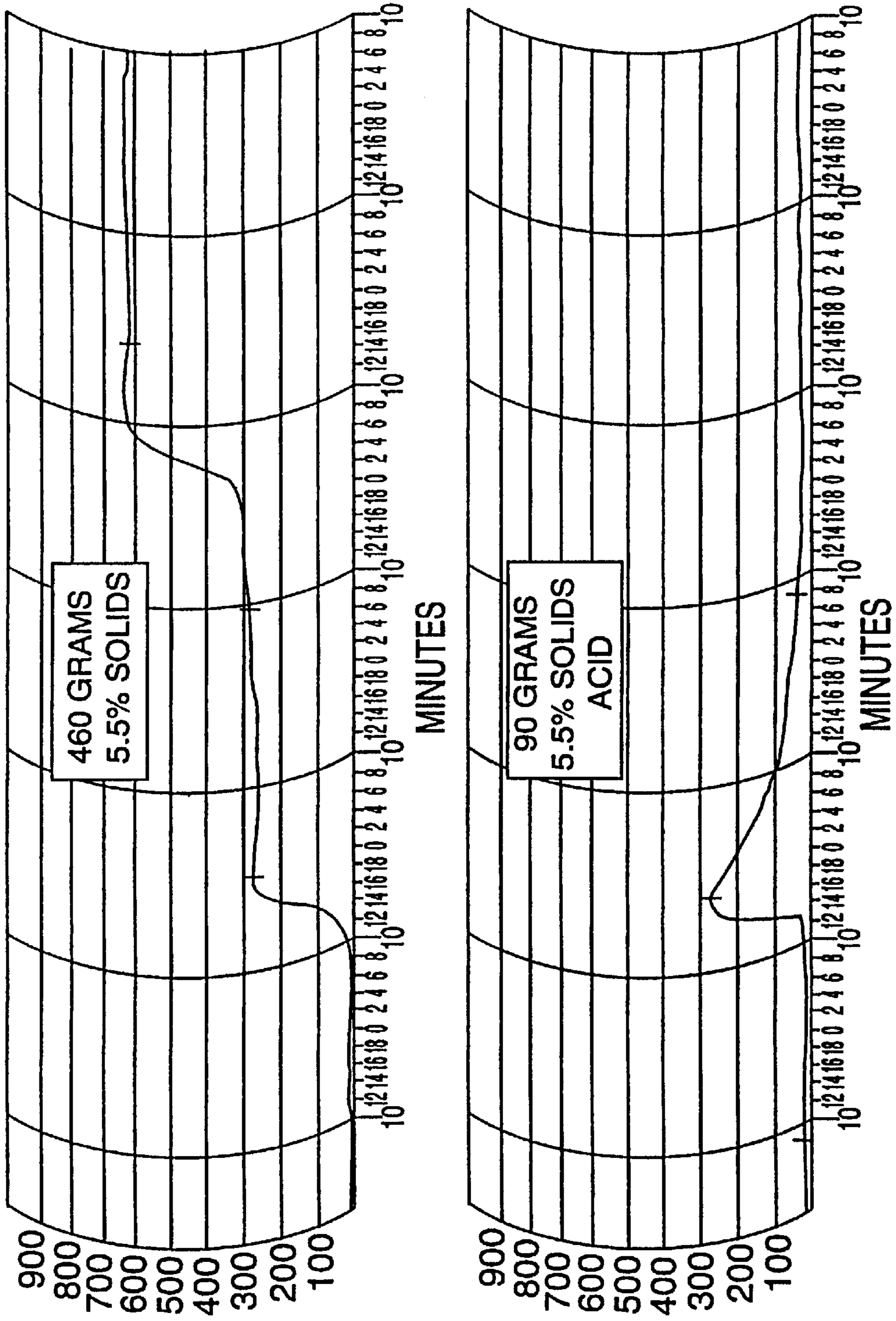


FIG. 3A

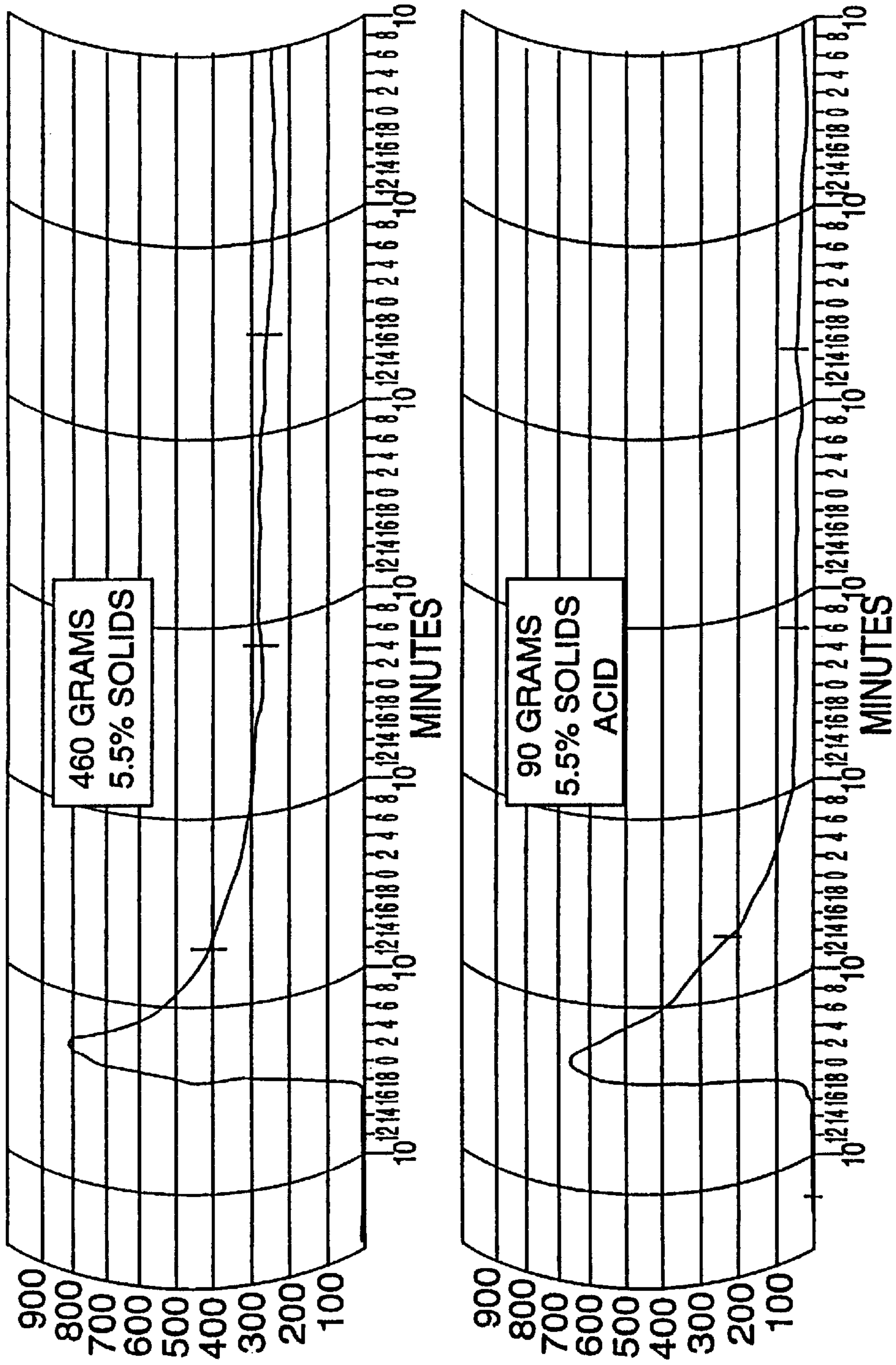


FIG. 3B

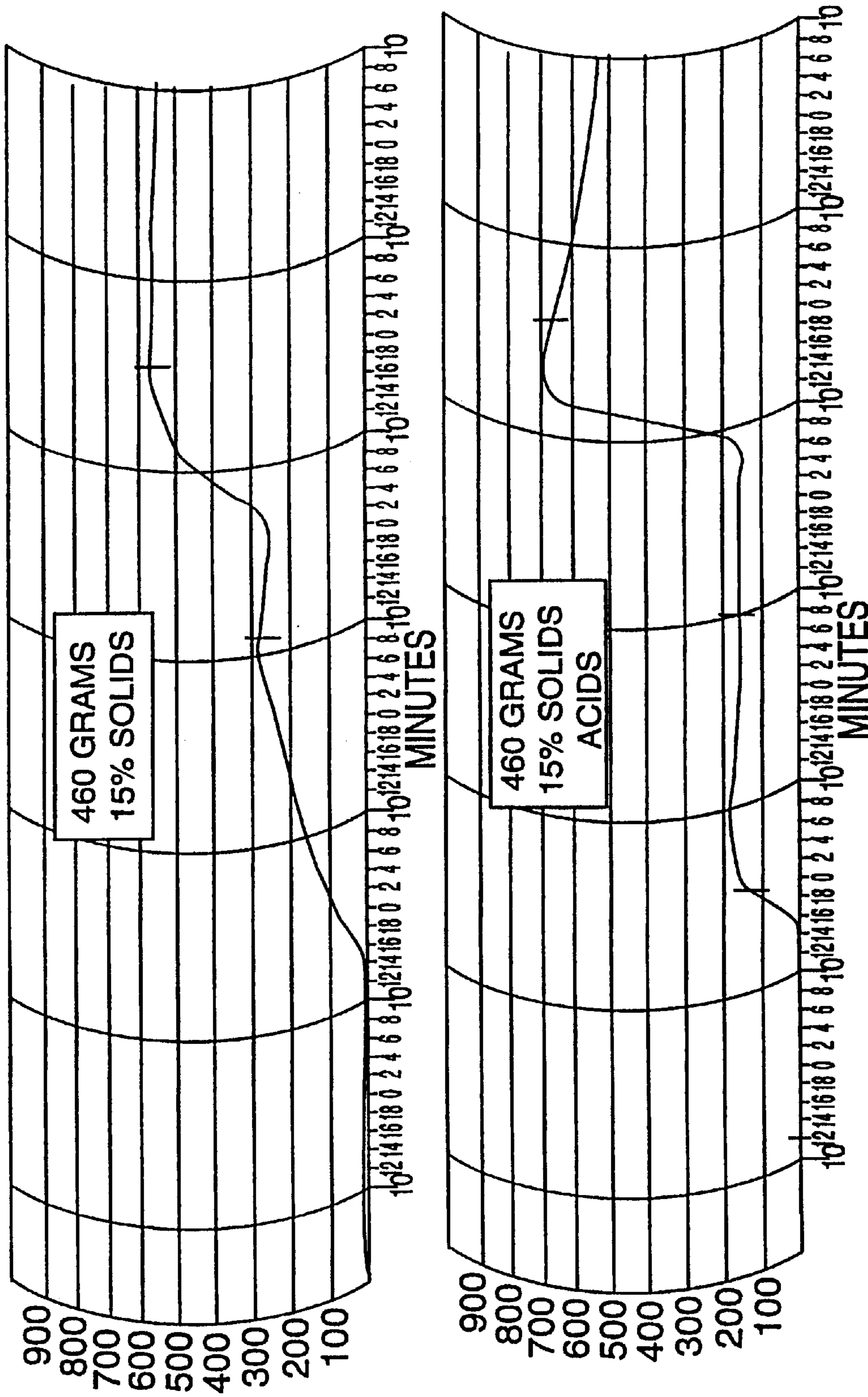


FIG. 3C

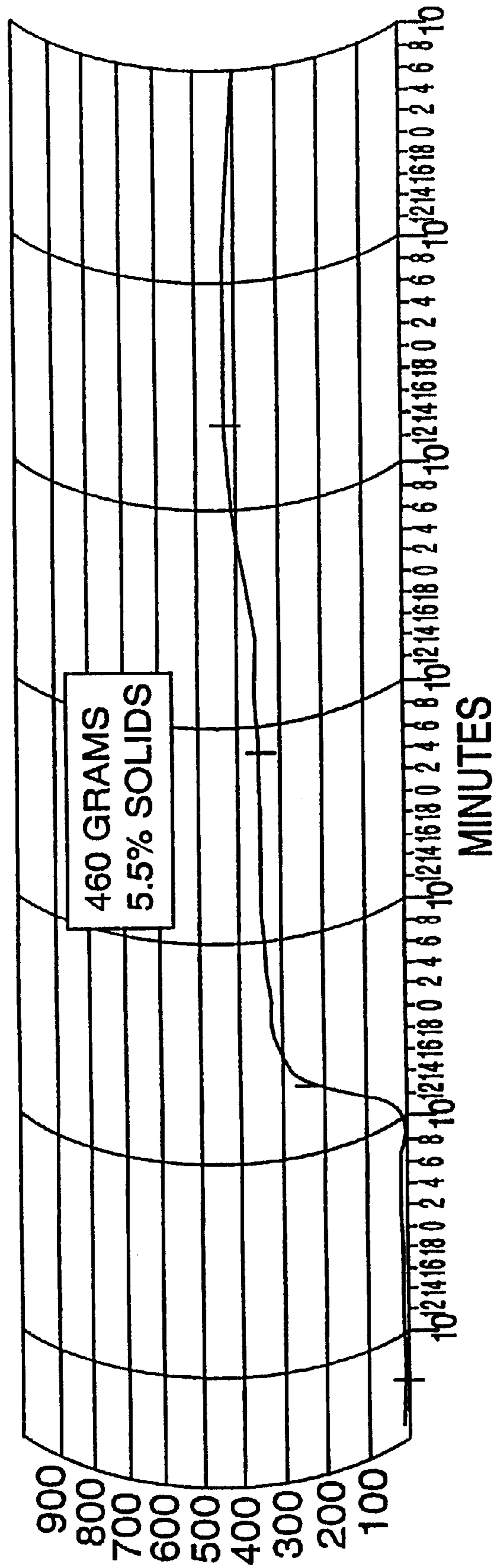


FIG. 3D

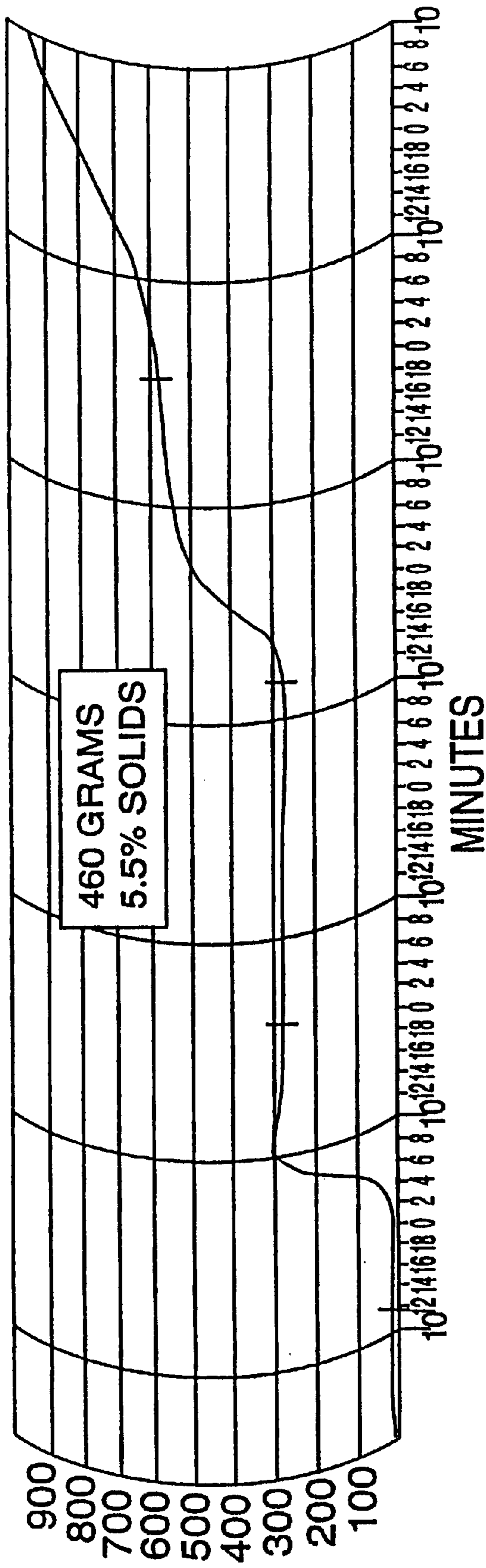


FIG. 3E

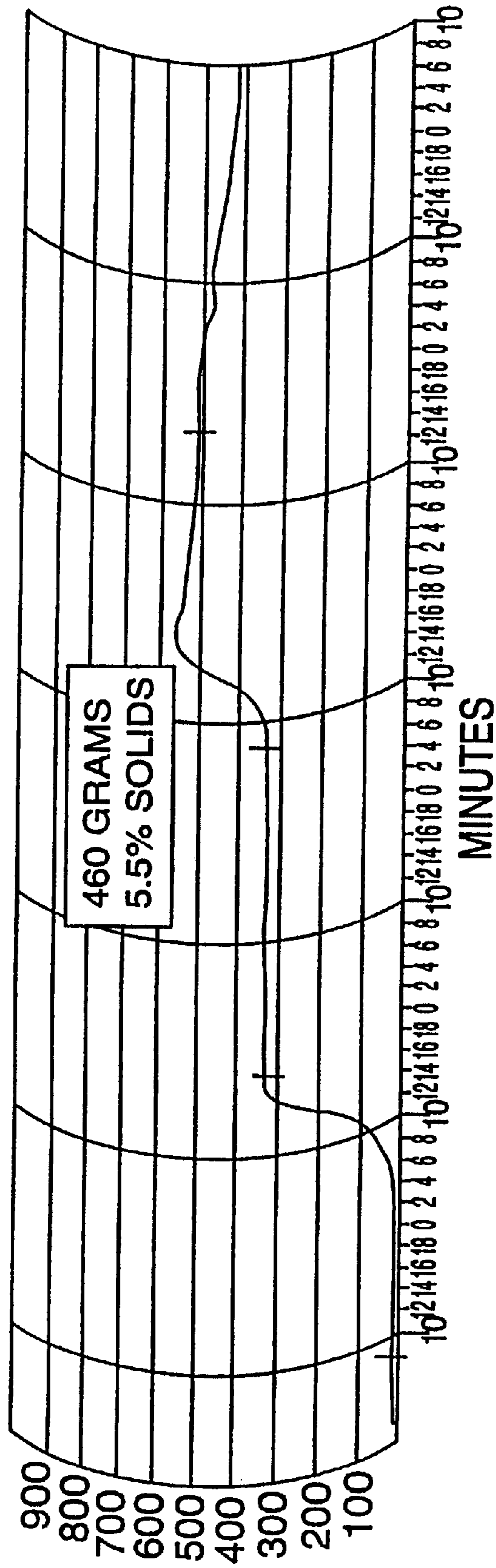


FIG. 3F

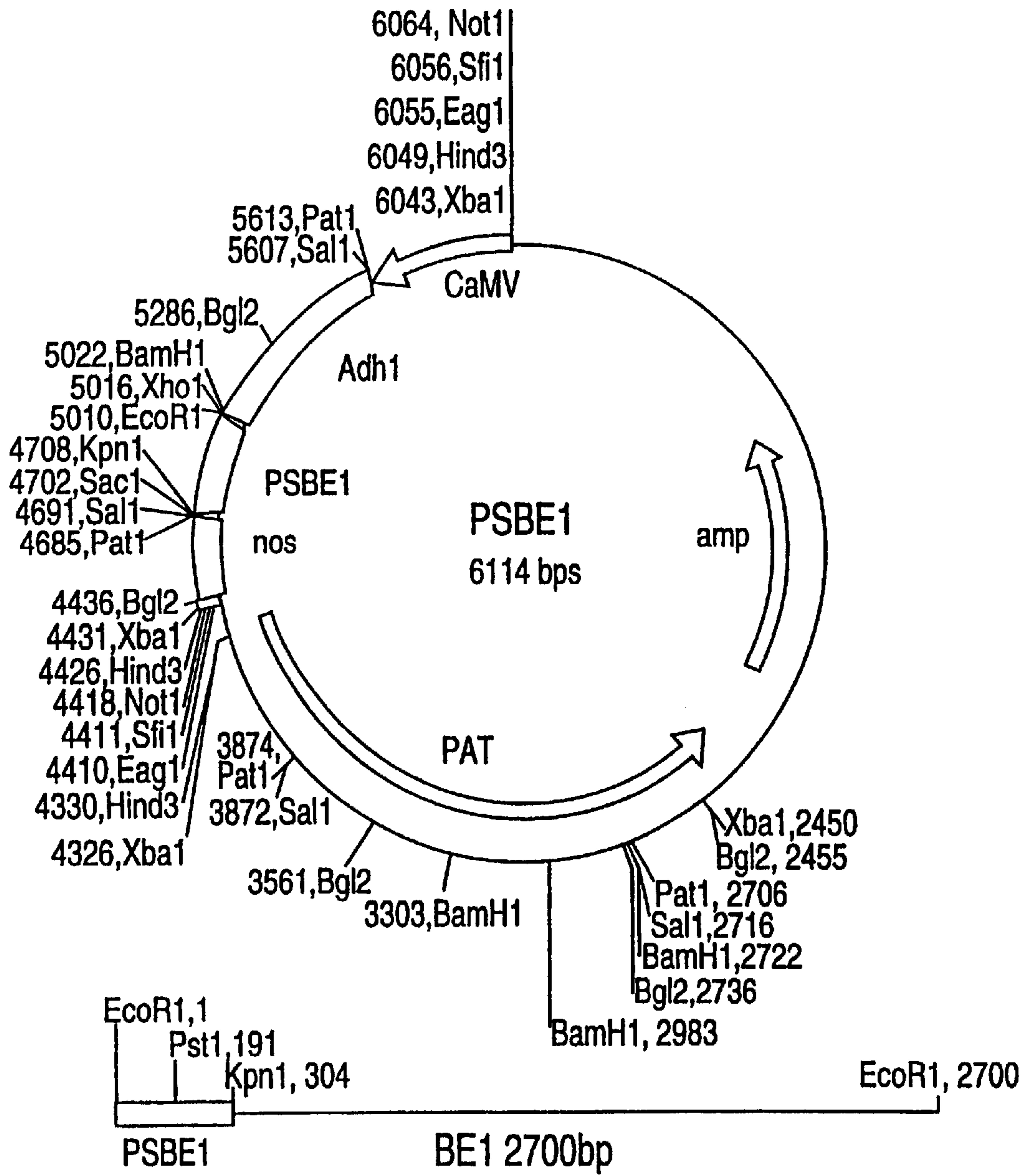


FIG. 4A

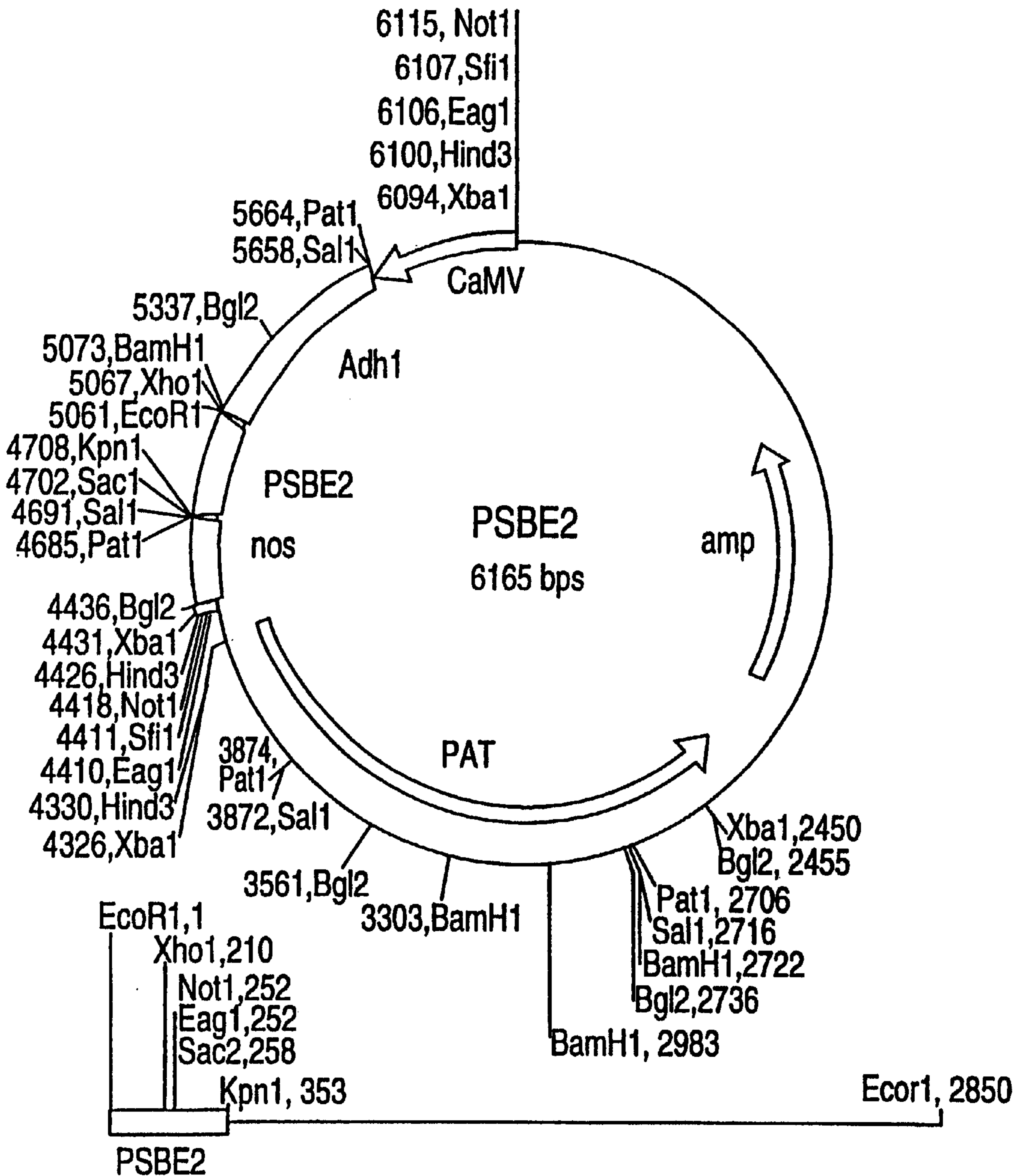


FIG. 4B

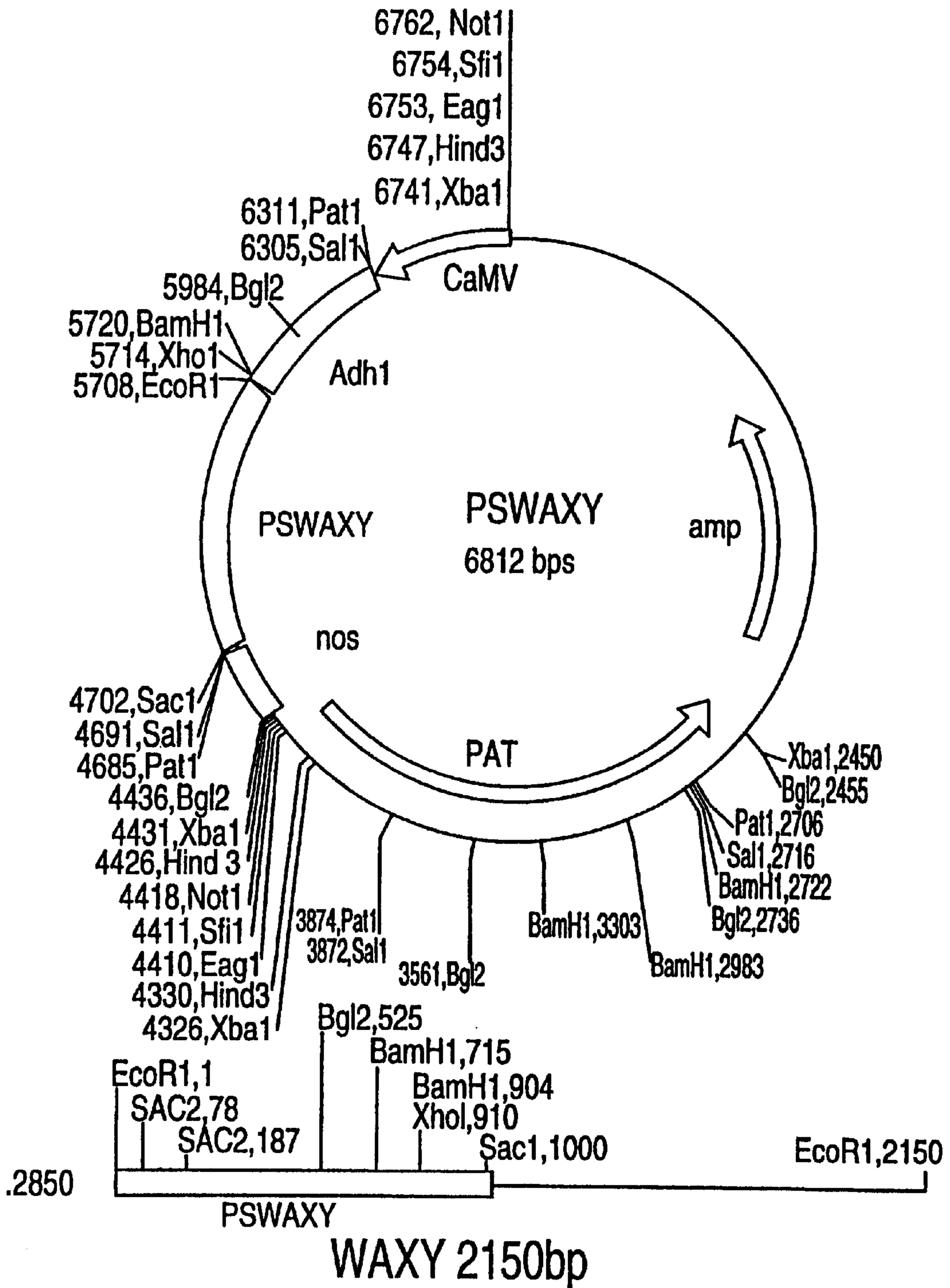


FIG. 4C

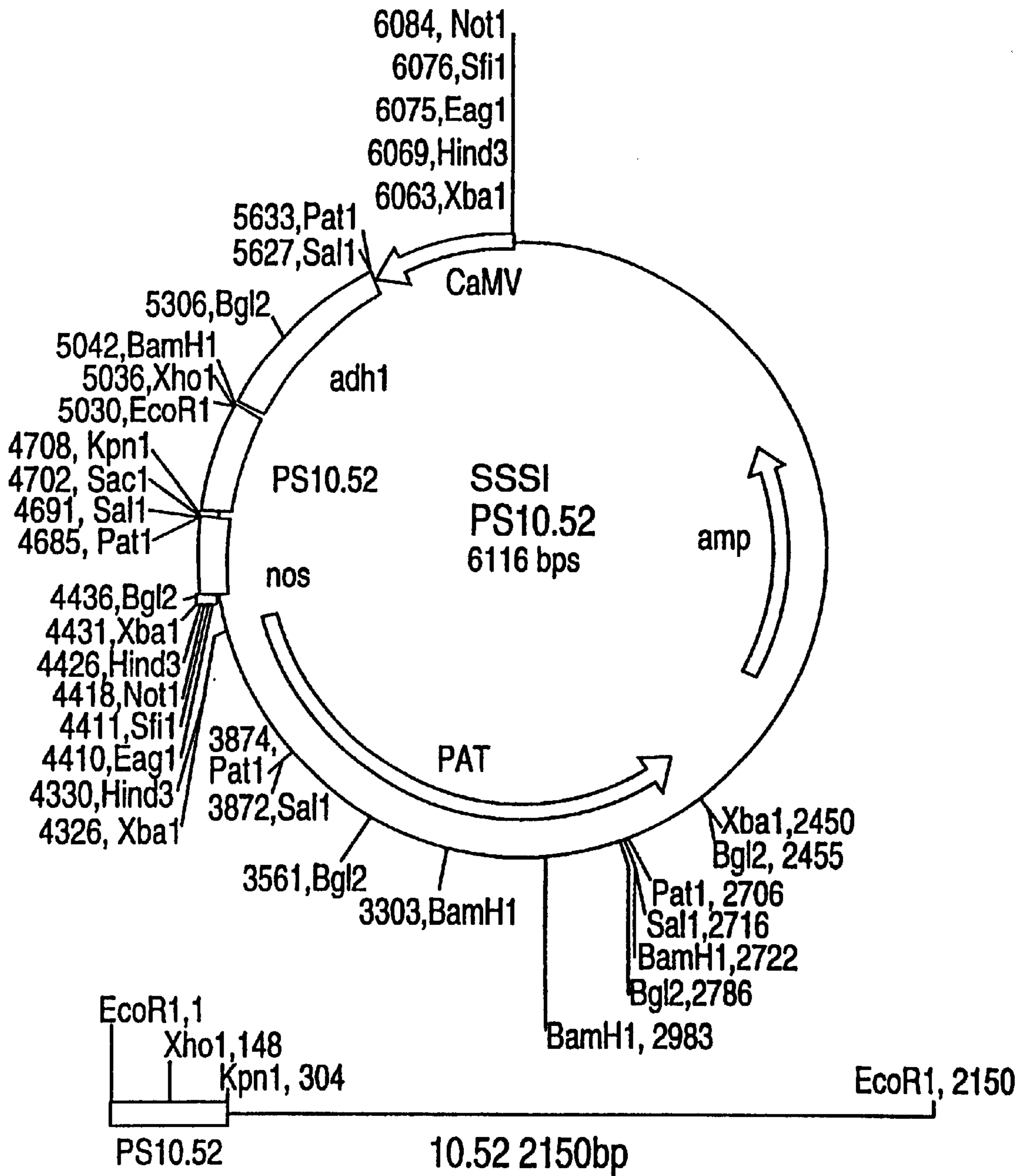


FIG. 4D

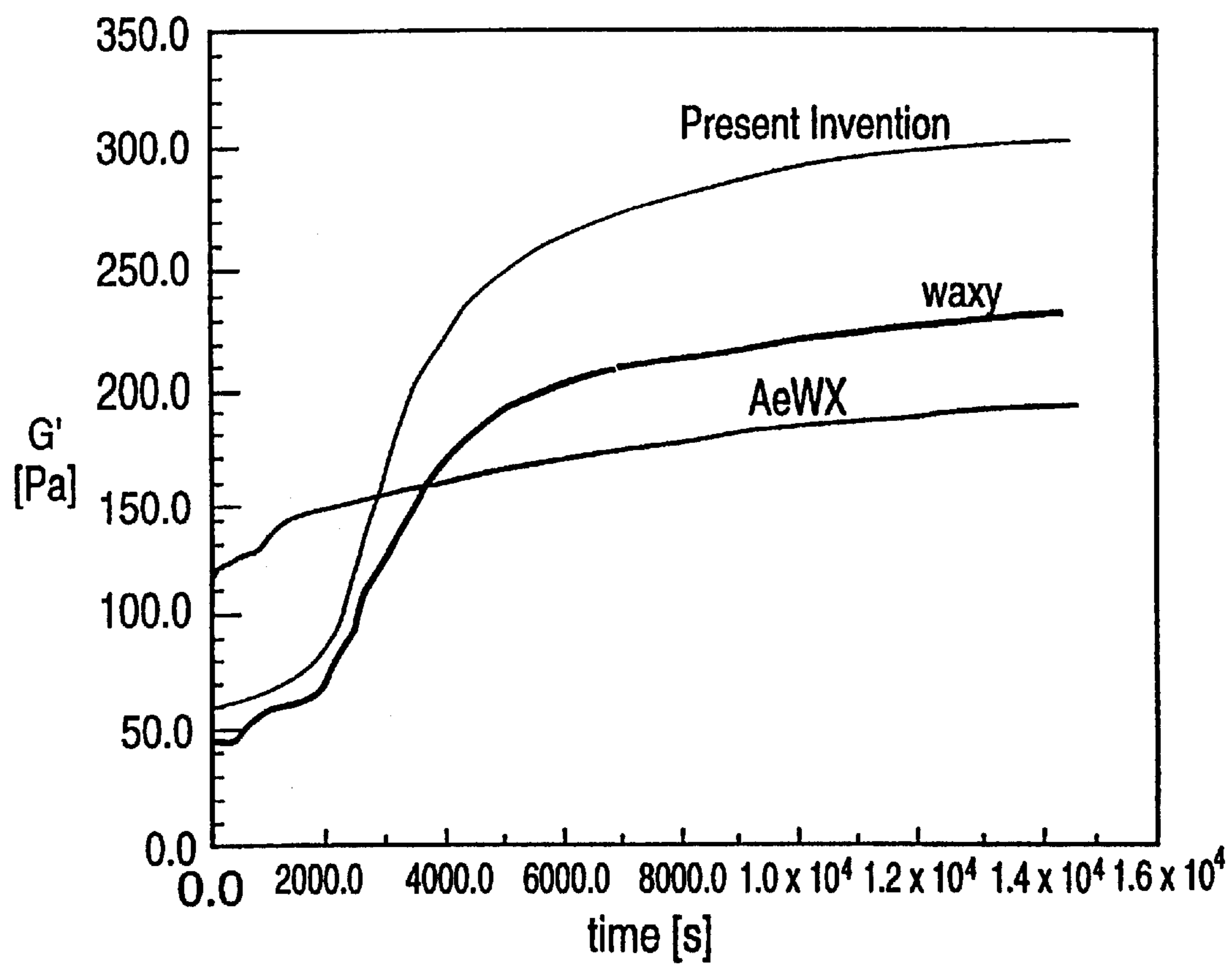


FIG. 5

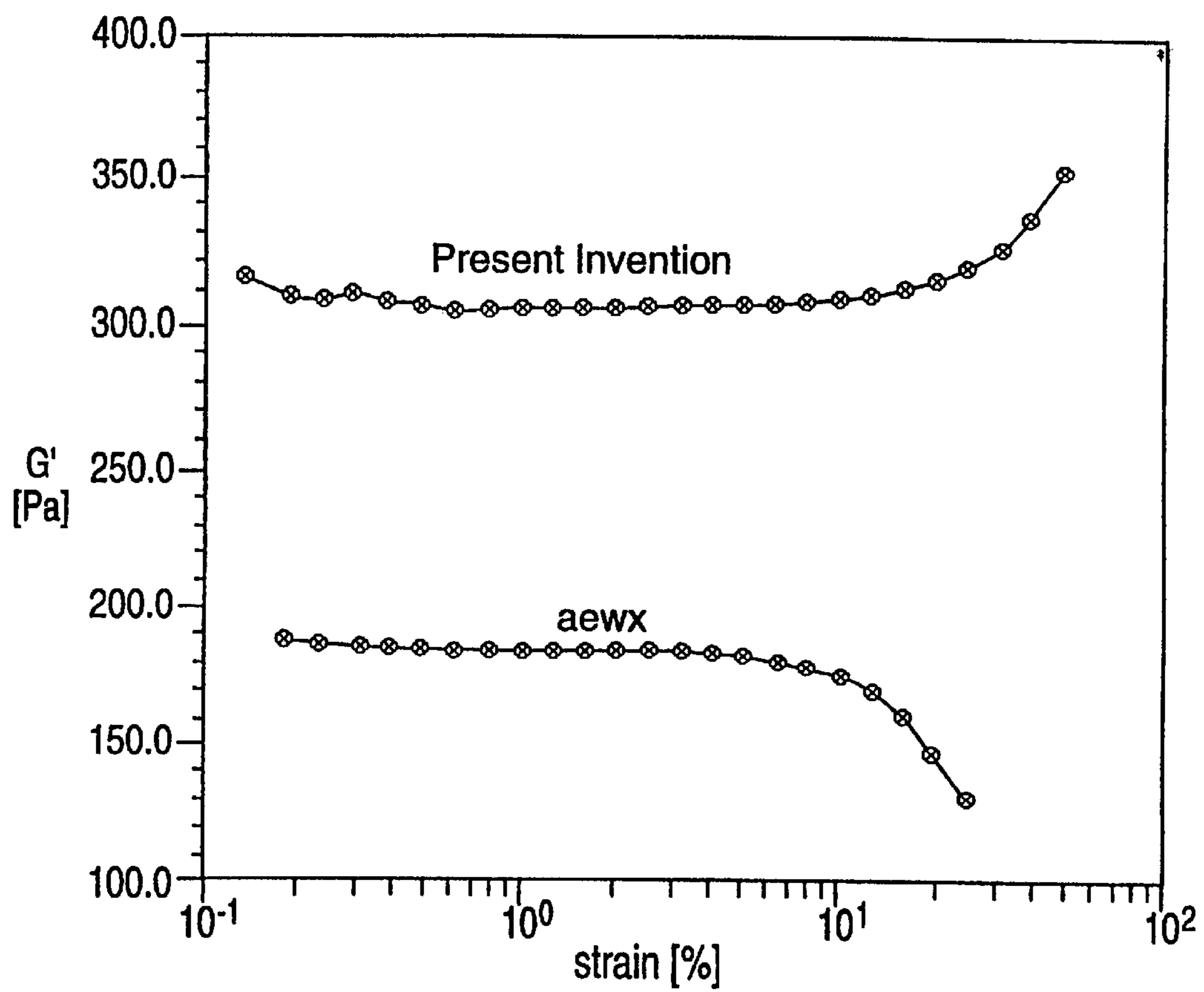


FIG. 6

PLANTS AND PROCESSES FOR OBTAINING THEM

This application is the national phase of international application PCT/US95/07828, filed Jun. 20, 1995 which designated the US.

FIELD OF THE INVENTION

The invention relates to a transgenic or mutated plant having genomic material which alters the normal starch synthesis pathway within the plant. More specifically, the present invention relates to a plant having a genotype which creates new forms of starch in significant quantity. Particularly, the invention relates to grain having an embryo with a genotype heterozygous for two or more wild type genes (for example, Aa/Bb) and an endosperm having a genotype heterozygous for such genes (for example, AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB) and the starch produced therefrom.

Such grain are produced by pollinating a plant having a genotype homozygous recessive for at least one gene and wild type for another gene (for example, aa/BB) with pollen from another plant having a genotype homozygous recessive for at least one other gene and wild type for the other gene (for example, AA/bb).

BACKGROUND OF THE INVENTION

Most plants produce and store starch. These plants have a starch synthesis pathway for starch production. The amount of starch produced varies with the type of plant. The most commonly known starch producing plants are the cereal grains. These cereals include rice, maize, sorghum, barley, wheat, rye, and oats. Additionally, the potato family, including the sweet potatoes and certain fruits, like the banana, are known as starch producing.

Starch is an important end-product of carbon fixation during photosynthesis in leaves and is an important storage product in seeds and fruits. In economic terms, the starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories.

Starch from plants is used in various ways. For example, it can be extracted and used for cooking and food processing. Starch can be left in the grain or plant and used for animal and human consumption. Starch can also be used in the distillation process for processing alcohols, for example, starch can be converted into ethanol. Additional starch can convert to high-fructose syrup and other industrial components.

Starch is defined in the dictionary as a granular solid which is chemically a complex carbohydrate which is used in adhesives, sizes, foods, cosmetics, medicine, etc. More generally, starch is comprised of amylose and amylopectin. Amylose and amylopectin is synthesized in the plastid compartment (the chloroplast in photosynthetic cells or the amyloplast in non-photosynthetic cells). Different plants generate differing proportions of amylopectin and amylose. Furthermore, the different branching patterns of amylopectin and different chain lengths of amylose and amylopectin chains gives rise to different starch properties. Thus, the fine structure of amylose and amylopectin is different in different plants so that the branching patterns and chain-lengths vary considerably resulting in new and novel properties which are useful in different applications. Until now there have been four ways of making starches with special properties: (i) using starches extracted from different plant species, (ii)

using starches extracted from mutant lines of particular plants, (iii) using natural and mutant starches which had been chemically modified, and (iv) using natural and mutant starches which had been physically modified. In all cases the new starches were valuable because of the special properties provided for by the new starch type.

It is known that mutant genes in plants affect the properties of the starch. A variety of starch related mutant genes in maize have been identified and some have been cloned. These mutant genes were named according to the physical appearance (phenotype) of the maize kernel or the properties of the starch. These recessive mutant genes include waxy (wx), sugary (su) [which includes but is not limited to sugary-1 (su1), sugary-2 (su2), sugary-3 (su3), sugary-4 (su4)] dull (du), amylose extender (ae), horny (h), shrunken (sh) which includes, but is not limited to, shrunken-1 (sh-1), shrunken-2 (sh-2). Some of these recessive gene mutants produce an isoform of a known enzyme in the starch synthesis pathway. The recessive mutant alleles of these genes result in a complete or nearly complete reduction in the activity of a specific isoform of one enzyme (hereinafter defined as complete reduction of enzyme isoform activity) in the pathway when homozygous in a plant or when expressed in sufficient levels in a transgenic plant. This change in the starch synthesis pathway causes the formation of starches with different properties.

Several crop varieties are known which produce different types of starch. The type of quality of starch makes it suitable for certain purposes, including particular methods of processing or particular end-uses. Naturally-occurring maize mutants produce starches of differing fine structure suitable for use in various food products and other applications. Although known mutants produce altered starch, some of these lines are not suitable for crop breeding and/or for the farmers' purposes. For example, they can give relatively poor yields, and/or are difficult to process and/or can have poor germination.

In order to generate different starches, single and double mutant plants have been bred. A single mutant is a plant that is homozygous for one recessive mutant gene. For example, waxy maize, waxy rice, waxy barley, and waxy sorghum have the homozygous mutant waxy (wx) gene. Whilst starches from waxy genotypes have very little or no amylose, another mutation known as amylose extender (ae) results in starch with high amylose. A double mutant is a single plant that has homozygous (or full expression) of two recessive mutant genes. For example, the wxfl1 double mutant is taught in U.S. Pat. No. 4,789,738. Many other novel starches have been provided in other starch patents in which double or triple mutants are generated (for example, U.S. Pat. Nos. 4,789,557, 4,790,997, 4,774,328, 4,770,710, 4,798,735, 4,767,849, 4,801,470, 4,789,738, 4,792,458 and 5,009,911 which describe naturally-occurring maize mutants producing starches of differing fine structure suitable for use in various food products). The present invention is highly surprising in light of these applications because it produces altered starch and does not require double or triple mutants.

Normal starch is defined as starch which is not chemically modified (by people) or which is produced from a plant that has the expected genes (wild type) regulating the starch synthesis pathway. For ease of reading, double lower-case letters, for example aa, shall refer to a homozygous recessive mutant gene, double upper-case letters, for example AA, shall refer to a homozygous non-mutant gene (wild type), and one upper-case and one lowercase letter, for example Aa, shall refer to a non-homozygous set of genes, one

mutant, one non-mutant. Different letters in the same size shall mean different genes; "aa/bb" would be a double mutant; "aa/bB" would be a single homozygous mutant gene and a heterozygous mutant gene in the genome of the plant. For purposes of this application, the order of any three letters on one side of the slash can be interchanged and will not define the parent that donated the gene. For example, AAa/bbB is defined to be equivalent to aAA/bBb, AaA/Bbb, AaA/bBb, aAA/Bbb, and the like.

Although maize plants and the embryo are diploid, maize endosperm is triploid. The endosperm genotype has two gene doses which are inherited from the female plant portion and one gene dose which is inherited from the pollen or male plant portion. Thus, if a single mutant plant "aa" is used as the female and crossed to a non-mutant plant "AA" male, then the endosperm in the kernel of this female plant would be "aaA". If a non-mutant plant "AA" is crossed to a mutant plant "aa" with the non-mutant as the female, the endosperm on the kernel of the female plant will be "Aaa", because two gene doses come from the female and one from the male plant. Classic teaching is that the mutant gene is recessive and the non-mutant is dominant; therefore, the starch produced by a plant having the following gene doses in the endosperm "aaA" or "AAA" or "AAa" results in the normal starch in the expected amounts. However, the endosperm of a homozygous mutant plant "aa" acting as the female crossed to a homozygous mutant plant "aa" acting as the male plant results in the endosperm having the gene dosage "aaa". This endosperm gives starch with different properties from normal starch. Likewise, the starch from a double mutant having of an endosperm which is "aaa/bbb" shows differences in starch properties from normal starch. These starch differences are useful in that they can replace chemically modified starches or be used with or in foodstuffs or as grain in alcohol production or in general starch industrial applications.

Clearly, it appears that production of grain having starch with different physical properties of starch requires the crossing of two mutated plants to generate grain which is homozygous recessive for both genes. Mutant plants are less predictable than standard plants.

There is a recurrent problem with the production of grain and extraction of starch from double mutant hybrids and/or inbreds and some single mutants. The amount of starch produced is usually less than the amount of starch produced by the non-mutant plant, there is also a loss in starch granule size and/or starch granule integrity. This problem with known double mutant lines which produce structurally-altered starch in which the quantity of starch produced in the crop is relatively low can result in poor germinability of the seed. Furthermore, the reduced starch yield of the seed appears to be unavoidable since the mutations cause the normal starch synthesis functioning of the cells to be disrupted. There remains a need for a way to produce grain having structurally altered starch structurally altered starch or altered properties without a significant loss of yield or reduced starch granule size or integrity.

SUMMARY OF THE INVENTION

A first object of the present invention is to provide a method of developing hybrid plants having altered complex carbohydrate content of the grain which does not require the crossing of double mutant inbreds.

An object of the present invention is to provide plants that produce grain having altered starch properties.

Another object of the present invention is to provide transgenic plants that produce grain having altered starch properties.

Yet another object of the present invention is to provide a maize plant that produces both altered starch and larger quantities of starch than the associated mutant plants produce.

5 Still another object of the present invention is to provide new plants which contain genes which produce incomplete reduction of the activity of at least two isoforms of the specific enzymes in the starch synthesis pathway of the plant. A further object of the present invention is to provide 10 maize plants which have endosperms with the genotype "AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB".

Yet another object of the present invention is a plant producing the following endosperm genotype "wxwxWx/AeAeae".

15 Still an additional object of the present invention is to provide the altered starch which can be produced by plants having the genotype "AAa/BBb or AAa/bbB or aaA/BBb or aaA/bbB".

20 Yet another object of the present invention is to provide novel uses of the starch obtained from the Maize plants of the present invention.

The present invention broadly covers a method of producing intermutants generally which have the endosperm genotype of AAa/bbB and certain intermutants including 25 endosperm which is waxy, waxy, amylose extender (wxwxWx/AEAEae). The method of producing grain with altered starch qualities includes the steps of planting the female acting parent which is capable of flowering. The female parent having substantially complete reduction of at least one specific isoform enzyme in the starch synthesis pathway. This can be by a homozygous recessive mutant or by the partial down regulation of the wild type gene through the use of a cloned gene using techniques generally known 30 as antisense or co-suppression or sense-down regulation. Additionally the female has incomplete reduction of at least one specific isoform enzyme in starch synthesis pathway. This can be by a heterozygous recessive mutant gene or partial down regulation. Regardless of how the female is produced it should only act as the female portion. To assure this a step includes eliminating the first parent's capability to produce pollen. The method includes the step of pollinating the female acting parent with the pollen of the male acting parent which is a non-mutant parent. Harvesting the grain 35 produced by said first parent. Additionally, the method can include the extraction of starch from the grain.

This invention also encompasses a plant having genomic material which includes genes which give incomplete reduction of the activity of at least two specific isoforms of the enzymes in the starch synthesis pathway of said plant And the starch it produces which has altered structure when compared with the starch formed by a similar plant as described but which comprises genomic material which does not form isoforms of the enzymes in the starch synthesis 40 pathway of the plant.

A plant which forms said starch in grain such as cereal grains. Grain produced by a female plant having for example a waxy genotype (wxwx) crossed with a male plant having for example an amylose extender genotype (aeae) in which the genotype of the endosperm of the grain is wxwxWx/AeAeae.

In other words, the present invention is a starch producing plant comprising genomic material which includes genes which give incomplete reduction of the activity of at least 45 two specific isoforms enzymes in the starch synthesis pathway of said plant whereby said plant produces substantially more starch than said plant would produce if said genes gave

complete reduction of the activity of the same two specific isoforms of the enzymes within the starch synthesis pathway.

Grain having an endosperm that has two genes which contain one gene dose of recessive mutant gene and two doses of wild type; and, having two gene doses of recessive mutant and one dose of wild type. Within this description the invention encompasses grain having endosperm genotypes of $wxwxWx/AeAeae$, or $aeaeAe/WxWxwx$, or $wxwxWx/DuDudu$, or $duduDu/WxWxwx$, or $aeaeAe/DuDudu$, or $duduDu/AeAeae$, or $wxwxWx/SuSusu$, or $susuSu/WxWxwx$, or $aeaeAe/SuSusu$, or $susuSu/AeAeae$, or $duduDu/SuSusu$, or $susuSu/DuDudu$ and the like.

The starch from a grain having a genotype of $wxwxWx/AeAeae$. The starch from a grain having a genotype of $Aeaeae/WxWxwx$.

A female plant having a diploid genotype of aa/BB and having a triploid genotype of aaA/BBb where a is a recessive mutant gene and A is the wild type gene, and b is a recessive mutant gene and B is the wild type gene such that the starch is altered from the normal starch where a and b can be selected from ae , wx , sh , bt , h , su , fl , op and B and A can be selected from Ae , Wx , Sh , Bt , H , Su , Fl , Op .

The starch obtained in accordance with the present invention produces a strong resilient gel which clears from the mouth uniquely fast. The starch of the present invention produces a gel with a unique and distinctive texture compared to conventional starches. The unique and distinctive texture makes the starch of the present invention suitable as a replacement for conventional gelling gums such as natural gums and gelatin, in whole or in part in food formulations. The starch of the present invention has also been found to produce a more resilient gel than common starch. Furthermore, it has been found that cornstarch produced from maize produces a gel which has improved clarity compared to a gel made from a common starch. Such improved clarity is visible to the human eye and lends itself to a more appetizing foodstuff.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described, by way of illustration, by the following description and examples with reference to the accompanying drawings of which:

FIG. 1 is a graph of enzyme activity for different gene dosages of a single mutant;

FIG. 2a is a graph of the DSC scan of waxy, amylose extender and common maize;

FIG. 2b is a graph of the DSC scan of a double mutant ($aeaeae/wxwxwx$);

FIG. 2c is a graph of the DSC scan of starch from an intermutant ($aeaeAe/wxwxwx$).

FIG. 2d is a graph of the DSC scan of starch from another intermutant ($wxwxWx/AeAeae$).

FIG. 3a is a graph of Brabender data of common starch in various pH.

FIG. 3b is a graph of Brabender data of waxy starch in various pH.

FIG. 3c is a graph of Brabender data of 70% amylose starch in various pH.

FIG. 3d is a graph of Brabender data of double mutant starch in various pH.

FIG. 3e is a graph of Brabender data of a first intermutant starch in various pH.

FIG. 3f is a graph of Brabender data of a second intermutant starch in various pH.

FIG. 4a is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of branching enzyme I.

FIG. 4b is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of branching enzyme II.

FIG. 4c is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels of bound starch synthase (waxy).

FIG. 4d is a schematic showing the design and restriction enzyme sites of plant transformation vectors used to alter gene expression levels soluble starch synthase.

FIG. 5 is a plot of the elastic modulus (G') over time comparing a gel made from the starch of the present invention to a gel made from an $aewx$ starch and a gel made from a waxy (wx starch); and

FIG. 6 is a plot of the elastic modulus (G') plotted against strain for both the present invention and an $aewx$ starch.

DETAILED DESCRIPTION OF THE INVENTION

Broadly then the present invention is an improved crop line which has manipulated expression of at least two starch-synthesizing enzymes which alter the amount and type of starch, and, consequently, alters the grain produced by the plant.

It has been discovered that plants which contain at least two genes which partially down regulated or reduce the activity of specific isoforms of enzymes in the starch synthesis pathway will surprisingly produce significant amounts of starch in the grain and will produce altered starch types.

Specialty maize or mutant plants differ from "normal" maize because of its altered endosperm. The changed endosperm gives rise to a high degree of starch branching, or changed sugar content, or different kernel structure. The endosperm of course is formed by the sperm and ovule, and the selection of both parents effects the endosperm's makeup.

The present invention can be formed by two principle methods. The invention can be formed within a selected crop species by the use of mutant breeding. And the invention can be formed in various plants by the use of transformation of the plants with genes which partially down regulate two or more enzymes in the starch synthesis pathway. More particularly, down regulation of one of the isoforms of the enzyme in the starch synthesis pathway to approximately $\frac{1}{3}$ of the normal activity and $\frac{2}{3}$ of the normal activity in the other isoform enzyme or down regulation of both isoform enzymes in the starch synthesis pathway approximately $\frac{2}{3}$ of the normal activity. Each of these methods has its own advantages.

First, the use of mutants to develop unique grain and starches in cereal crops is widely known. However, the present invention is highly unique and surprising because it was expected to produce grain having normal starch characteristics. The following table explains how unexpected the present invention is.

TABLE 1

Genotype of Parents (female 1st)	Genotype of endosperm	Type of Starch	
		Starch	Yield
Wild-Type AA*AA Gene dosage	AAA	Normal	Normal
AA*aa	AAa	Normal	Normal
aa*AA	aaA	Normal	Normal
Single mutant aa*aa	aaa	Altered	Lowered
Double mutant aa/bb*aa/bb	aaa/bbb	Altered	Lowered
<u>PRESENT INVENTION - EXPECTED RESULTS</u>			
<u>Intermutant</u>			
aa/BB*AA/bb	aaA/BBb	Normal	Normal
AA/BB*aa/bb	AAa/BBb	Normal	Normal
aa/bb*AA/BB	aaA/bbB	Normal	Normal
aa/bb*aa/BB	aaa/bbB	Normal	Normal
aa/BB*aa/bb	aaa/BBb	Normal	Normal
<u>PRESENT INVENTION - ACTUAL RESULTS</u>			
<u>Intermutant</u>			
aa/BB*AA/bb	aaA/BBb	Altered	Medium to
AA/BB*aa/bb	AAa/BBb	Altered	high ~70%
aa/bb*AA/BB	aaA/bbB	Altered	of normal
aa/bb*aa/BB	aaa/bbB	Altered	
aa/BB*aa/bb	aaa/BBb	Altered	

aa = mutant gene (homozygous)

AA = wild type (or non-mutant homozygous gene)

* = signifies a cross-pollination between two varieties

aa/bb = two mutant genes (both homozygous)

Clearly, since the genotype of the endosperm of the present invention shown in the table did not have completely recessive genes, the starch yield and structure was expected to be normal. In fact, according to the present invention, the grain does not evidence normal starch structure altered starch. Historically, when altered starch is produced only small quantities are usually produced. The altered starch of the present invention was surprisingly produced in larger than expected quantities. Additionally, production of this starch is much simpler than the production of double mutant crops. Previously only single mutant hybrids have been used extensively for large-scale starch production. Previously, to develop the double mutants, both parents had to carry both mutations which requires significant research and development efforts and results in poor starch yield and poor seed germinability. Heretofore, only small-scale production of the double mutants has been possible.

The present invention encompasses a method of producing grain with altered starch qualities which includes the steps of planting a parent which is capable of flowering, this parent having substantially complete reduction of at least one specific isoform of an enzyme (A) in the starch synthesis pathway and having no reduction of at least one other specific isoform of an enzyme (B) in the starch synthesis pathway. The other parent has no reduction of one isoform of an enzyme (A) and substantially complete reduction of at least one other specific isoform of an enzyme (B) in the starch synthesis pathway. It is then necessary to eliminate said first parents capability to produce pollen and allow pollination to proceed from said second mutant parent, and finally harvesting the grain produced by said first parent. Additionally, the method can include the extraction of starch from the grain and using said starch as a specialty starch for a variety of uses for which it is shown to be valuable.

In order to prepare a sol in accordance with a the present invention, a slurry is prepared which comprises water and an

effective amount of starch of the present invention and the sol subject to a cooking step to form a paste. Generally, cooking entails raising the temperature of the slurry to above about the gelatinization temperature of the starch and subjecting the starch to enough shear such that the granules rupture and a paste is formed. It is not necessary that all the granules rupture. Preferably, the sol contains the starch of the present invention in the amount of about 1 to about 20% by weight total sol. The slurry is cooked at a temperature of about 90° C. and above to provide thickening characteristics prior to adding to the foodstuff. Cooking time is about 10 minutes. The sol in accordance with the present invention need not be cooked if the starch has already been subjected to a process which makes it cold water swellable. Cooking generally comprises raising the temperature of an aqueous slurry of the starch of the present invention to the gelatinization temperature of the starch and subjecting the starch to shear such that the starch granules rupture and form a paste.

A sol or a thickener composition of the starch of the present invention is added to a foodstuff in a conventional manner in order to provide the benefits of the starch of the present invention to the foodstuff.

In order to prepare the thickened foodstuff, a sol made in accordance with the present invention is combined with a foodstuff and the composition is cooked to the necessary degree to provide a thickened foodstuff. Conventional mixing is employed to combine the sol with the foodstuff. Cooking of the sol and foodstuff composition is also carried out in a conventional manner.

Alternatively, starch of the present invention is mixed with the foodstuff or a slurry comprising the starch of the present invention and water is mixed with a foodstuff and the resulting mixture is cooked to the desired degree to obtain a thickened foodstuff. When the starch itself or a slurry containing the starch itself is mixed with a foodstuff, the resulting mixture must be cooked in order to provide a thickened foodstuff. The mixing as well as the cooking is accomplished in a conventional manner. Cooking is carried out at a temperature of about 90° C. and above. Cooking time is about 10 minutes but may vary depending on the amount of foodstuff present and the amount of shear that the mix is subject to during cooking.

Such a thickener composition can provide considerable economic advantage to the user. Those familiar with the art have long used a variety of gelling gums for their clean breaking texture. Application of the present invention have included but are not limited to gum candies, gelled desserts, glazes and spreads and can be used to replace conventional gelling gums such as kappa carrageenin, agar, pectin, or gelatin. These conventional gelling gums can be quite expensive however, and have other disadvantages including the presence of off-flavors, lack of heat or acid stability, limited availability, or lack of Kosher approval. It has been found that the starch of the present invention can replace all or a portion of these conventional gelling gums.

In order to replace a gelling gum in food formulations, a weight ratio of about 1:1, starch of the present invention:gelling gum, can be employed. Larger or smaller amounts of the starch of the present invention may be used to replace a gelling gum. Such gelling gums include gelatin, pectin, carrageenin, gum arabic, tragacanth, guar, locust bean, xanthan, agar, algin and carboxymethyl cellulose.

Naturally, the starch of the present invention can be used in any food formulation, where there is a need to provide gel characteristics and a clean break from the mouth. For example, the starch of the present invention can be used in a food formulation which had heretofore employed a common starch, thereby providing the food with improved

properties, i.e. clean break when compared to the same food formulation using a common starch.

The clean break of a gel made with the starch of the present invention is useful in a variety of food applications. The clean break of the starch gel has value in a variety of bakery applications, for example cream or fruit fillings for pies such as lemon, banana cream or Bavarian cream; and in low or reduced fat high solids fruit centers for cookies, for example, in fig bars. The starch of the present invention also creates an improved texture in mousses, egg custards, flans and aspics.

The term starch as used in the specification and claims means not only the substantially pure starch granules as extracted from a starch bearing plant but also grain products of the starch granule such as flour, grit, hominy and meal.

The following examples of the present invention are given for illustrative purposes only. These examples are not intended to limit the type or the uses of the present invention. The present invention or the grain or starch or sugar thereof can be useful in, but may not be limited to, the preparation of foodstuff, paper, plastics, adhesives, paints, production of ethanol and corn syrup products.

EXAMPLE 1

The physical properties of various embodiments of the present invention (similar genotypes are from different maize crosses). These tables display data well known to

people skilled in the arts of evaluating new and novel starches. Data on moistures, and percent oil, protein, solubles and starch are useful in evaluating yield and milling potential. Starch DSC (Differential Scanning Calorimetry) data are valuable for evaluating starch cooking and gelatinization properties. Starch particle size data are valuable for making decisions on starch milling and separation properties. Brabender and starch paste data are most important for evaluating a new starch's potential for improved food applications where particular starch thickening and pasting and gelatinization properties are mostly desired. Such data when interpreted as a whole collection of information enable one skilled in the art to decide whether to conduct further more detailed tests of the starches properties and potential.

Genotype	% Moisture	% Starch	% Protein	% Oil	% Solubles
aeae/wx	8.01	72.12	11.30	3.63	6.51
aeae/wx	8.00	71.34	11.74	3.79	6.41
aeae/wx	8.03	67.52	12.61	3.67	7.01
aeae/ae	8.13	60.11	14.59	6.60	10.14
su1/su1/wx	8.18	69.66	12.09	4.03	6.91
dudu/wx	7.98	71.10	11.57	3.92	6.61
dudu/su1	7.90	70.53	11.67	3.93	6.81
ffl/o	7.78	70.23	11.60	4.23	6.59
su1su1/du	7.81	69.00	12.20	4.77	7.98
su1su1/ae	7.88	69.03	12.27	4.88	8.02
su1su1/su2	7.95	69.67	12.28	4.55	7.49

TABLE 2

		Corn Data					
Entry	Background	S93 Row	% Moisture	% Starch	% Protein	% Oil	% Solubles
1	aeae/wx	4660	9.15	64.80	14.58	4.47	7.64
2	aeae/su	4636	9.27	66.25	14.77	4.54	7.40
3	aeae/du	4612	9.24	66.01	14.65	4.73	7.80
4	wx/wxae	4600	9.07	65.72	13.91	4.38	8.14
5	wx/wxsu	4648	9.06	66.03	14.79	4.08	7.83
6	wx/wxdu	4624	8.77	65.32	15.62	4.27	8.23
7	susuae	4594	8.85	64.83	13.11	4.71	8.49
8	susudu	4672	8.73	65.39	13.20	5.01	8.35
9	susudu	4618	8.77	65.15	14.52	4.35	8.26
10	single ae	4582	9.14	60.52	15.19	5.95	9.29
11	single wx	4654	8.83	65.44	14.04	4.77	9.43
12	single su	4630	7.75	54.98	14.25	6.50	9.78
13	duduae	4588	8.97	69.26	12.30	4.89	6.85
14	dudu/wx	4666	8.88	70.43	11.25	4.40	6.96
15	dudusu	4642	9.48	70.43	13.48	4.67	6.92
16	single du	4606	8.50	65.54	12.02	6.03	9.33

		Starch Data						
Entry	Background	S93 Row	% Moisture	% Starch	% Protein	% Oil	% Amylose	L-max
1	aeae/wx	4660	3.82	86.44	1.68	0.18	31.60	597.1
2	aeae/su	4636	6.51	86.95	1.56	0.20	31.74	603.9
3	aeae/du	4612	5.22	86.44	1.70	0.11	33.38	598.8
4	wx/wxae	4600	7.33	87.89	0.39	0.09	21.10	601.1
5	wx/wxsu	4648	7.27	85.87	1.32	0.09	24.68	591.7
6	wx/wxdu	4624	7.31	86.07	1.12	0.10	24.57	595.2
7	susuae	4594	8.19	86.32	0.93	0.07	29.00	604.5
8	susudu	4672	8.09	87.16	0.52	0.08	27.86	599.6
9	susudu	4618	7.67	84.20	0.60	0.12	30.82	601.1
10	single ae	4582	7.16	83.31	1.37	0.15	65.55	602.1
11	single wx	4654	8.30	85.16	0.51	0.14	0.43	xx
12	single su	4630	6.82	80.92	5.08	xx	29.32	598.4
13	duduae	4588	6.58	84.31	0.62	0.17	29.86	xx
14	dudu/wx	4666	11.08	84.77	0.48	0.16	28.74	xx
15	dudusu	4642	8.48	87.34	0.62	0.16	26.07	xx
16	single du	4606	8.36	80.70	0.46	0.08	37.88	608.9

TABLE 2-continued

(Starch Data)							
Entry	Genotype	% Moisture	% Starch	% Protein	% Oil	% Amylose	L-max
1	ae ae/wx	9.56	83.84	0.63	0.05	29.44	593.1
2	ae ae/wx	7.96	85.94	0.70	0.05	27.89	596.6
3	ae ae/wx	11.71	82.54	0.82	0.05	42.54	595.1
4	ae ae/ae	6.09	84.17	1.24	0.14	63.60	602.4
5	su1 su1/wx	4.37	90.00	0.56	0.09	28.36	600.0
6	du du/wx	5.95	88.50	0.61	0.09	27.98	596.9
7	du du/su1	6.93	82.66	0.69	0.10	29.98	600.8
8	fl fl/0	9.24	82.72	0.93	0.14	29.29	596.8
9	su1 su1/du	9.01	84.82	0.71	0.02	29.57	600.8
10	su1 su1/ae	7.78	84.81	0.63	0.03	29.90	600.8
11	su1 su1/su2	7.61	85.02	0.49	0.02	29.68	602.3
12	White waxy	18.34	94.94db	0.77	0.09	2.74	526.9

Starch DSC Data						
Entry	Genotype	Peak ° C.	Delta H J/g	Peek II ° C.	Onset ° C.	Endset ° C.
1	ae ae/wx	67.7	11.67	100.0	62.6	76.6
2	ae ae/wx	66.5	10.33	96.8	61.9	74.8
3	ae ae/wx	74.6	10.00	100.8	68.8	83.4
4	ae ae/ae	81.0	11.83	xx	66.0	107.4
5	su1 su1/wx	69.7	12.00	98.3	64.2	77.7
6	du du/wx	72.2	11.50	99.5	66.6	82.3
7	du du/su1	70.2	9.67	98.4	65.7	78.3
8	fl fl/0	71.4	12.33	100.9	67.1	80.7
9	su1 su1/du	68.8	7.83	99.5	63.2	79.1
10	su1 su1/ae	68.1	10.33	99.8	61.9	78.3
11	su1 su1/su2	67.4	12.00	96.1	61.3	79.5
12	White waxy	72.7	15.33	xx	66.1	82.6

Starch DSC Data							
Entry	Background	S93 Row	Peak ° C.	Delta H J/g	Peek II ° C.	Onset ° C.	Endset ° C.
1	aeaewx	4660	73.8	11.17	99.4	67.7	82.8
2	aeaewx	4636	73.0	11.50	101.0	66.6	83.4
3	aeaewx	4612	73.3	10.83	98.6	66.7	83.0
4	wxwsae	4600	72.9	12.83	99.9	67.4	81.7
5	wxwssu	4648	73.7	12.17	98.2	68.2	81.6
6	wxwsdu	4624	73.6	11.33	99.6	69.3	80.5
7	susuae	4594	72.0	10.33	97.7	67.2	79.7
8	susudu	4672	71.9	9.83	98.9	67.0	79.1
9	susudu	4618	72.5	10.33	97.7	68.4	79.8
10	single ae	4582	84.6	15.67	xx	68.7	106.9
11	single wx	4654	72.5	16.17	xx	68.1	81.4
12	single su	4630	70.9	15.50	100.8	63.6	77.2
13	duduae	4588	71.8	13.50	100.5	67.1	81.1
14	duduwx	4666	71.9	10.17	98.8	67.2	79.5
15	dudusu	4642	71.7	10.17	97.2	66.8	79.0
16	single du	4606	71.6	9.50	100.1	65.8	79.8

Brabender Data								
Entry	Background	S93 Row	Brabender	IR ° C.	HP BU	HF BU	CP BU	CF BU
1	aeaewx	4660	460 g/5.5%	65.0	315	285	940	940
2	aeaewx	4636	460 g/5.5%	81.5	225	225	470	430
3	aeaewx	4612	460 g/5.5%	84.5	180	180	410	380
4	wxwsae	4600	460 g/5.5%	74.0	360	355	590	460
5	wxwssu	4648	460 g/5.5%	74.0	325	325	540	470
6	wxwsdu	4624	460 g/5.5%	80.0	315	270	480	425
7	susuae	4594	460 g/5.5%	77.0	270	270	610	585
8	susudu	4672	460 g/5.5%	77.0	295	295	785	610
9	susudu	4618	460 g/5.5%	77.0	295	295	620	620
10	single ae	4582	460 g/12%	90.5	170	170	240	240
11	single wx	4654	460 g/5.5%	68.0	750	360	415	405
12	single su	4630	90 g/5.5%	93.5	35	35	40	40
13	duduae	4588	460 g/5.5%	83.0	225	225	545	520
14	duduwx	4666	460 g/5.5%	86.0	245	245	600	550

TABLE 2-continued

15	dudusu	4642	460 g/5.5%	84.5	270	270	620	585
16	single du	4606	460 g/5.5%	89.0	70	70	160	160

IR = initial rise
 CP = cooling peak
 CF = cooling final

		Brabender Data					
Entry	Genotype	Brabender	IR ° C.	HP BU	HF BU	CP BU	CF BU
1	ae ae/wx	460 g/5.5%	83.0	220	220	520	460
2	ae ae/wx	460 g/5.5%	89.0	220	220	540	510
3	ae ae/wx	460 g/5.5%	84.5	270	270	510	450
4	ae ae/ae	460 g/12%	90.5	490	490	1220	845
5	su1 su1/wx	460 g/5.5%	80.0	250	240	595	535
6	du du/wx	460 g/5.5%	83.0	280	250	560	470
7	du du/su1	460 g/5.5%	50.0/83.0	230	230	575	535
8	fl fl/0	460 g/5.5%	81.5	280	255	630	560
9	su1 su1/du	460 g/5.5%	84.5	200	200	500	460
10	su1 su1/ae	460 g/5.5%	86.0	205	205	455	415
11	su1 su1/su2	460 g/5.5%	84.5	180	180	420	385
12	White waxy	90 g/5.5%	68.0	830	220	310	270

		Starch Particle Size Data				
		S93	(Volume Distribution)			% Starch
Entry	Background	Row	Mode μm	Mean μm	Median μm	Recovery
1	aeaewx	4660	16.90	12.57	16.04	45.9
2	aeaewx	4636	16.63	11.82	15.23	57.9
3	aeaewx	4612	16.36	11.48	15.20	52.4
4	wxwsae	4600	17.43	11.62	16.53	41.5
5	wxwssu	4648	17.72	12.43	16.59	58.8
6	wxwsdu	4624	17.48	12.22	16.26	54.1
7	susuae	4594	16.36	11.96	15.66	55.5
8	susudu	4672	16.65	9.83	15.29	58.7
9	susudu	4618	16.36	11.82	15.61	55.9
10	single ae	4582	13.09	9.07	12.25	64.0
11	single wx	4654	18.03	11.99	16.85	55.7
12	single su	4630	7.43	5.16	7.95	5.6
13	duduae	4588	16.90	11.01	15.37	50.9
14	duduwx	4666	17.19	11.85	16.21	52.5
15	dudusu	4642	17.17	12.22	16.01	44.9
16	single du	4606	14.41	9.77	13.19	58.9

		Starch Particle Size Data				
		(Volume Distribution)			% Starch	
Entry	Genotype	Mode μm	Mean μm	Median μm	Recovery	
1	ae ae/wx	16.63	10.90	15.70	51.4	
2	ae ae/wx	16.12	10.44	14.92	52.8	
3	ae ae/wx	15.60	10.55	14.60	59.4	
4	ae ae/ae	11.53	8.40	11.30	63.7	
5	su1 su1/wx	14.88	10.24	14.21	52.5	
6	du du/wx	16.38	10.73	15.12	52.0	
7	du du/su1	15.87	11.03	14.98	53.2	
8	fl fl/0	16.12	10.73	15.35	69.3	
9	su1 su1/du	15.87	10.73	14.92	53.7	
10	su1 su1/ae	15.11	7.85	14.35	48.5	
11	su1 su1/su2	15.85	10.33	14.20	58.4	
12	White waxy	17.19	8.45	14.81	7.15	

		Starch Paste Data		
Entry	Genotype	Brookfield Viscosity CPS, 20 rpm	Gel-24 hr (grams)	Freeze Thaw Cycles
1	ae ae/wx	6,200	166.6	0
2	ae ae/wx	6,700	227.2	0
3	ae ae/wx	8,000	256.3	0
4	ae ae/ae	14,250	111.1	0
5	su1 su1/wx	6,800	173.4	0
6	du du/wx	6,000	141.4	0
7	du du/su1	7,100	215.4	0

TABLE 2-continued

Starch Paste Data					
Entry	Genotype	S93 Row	Brookfield Viscosity CPS, 20 rpm	Gel-24 hr	Freeze Thaw Cycles
8	fl fl/0	7,500	209.4	0	
9	su1 su1/du	6,300	219.7	0	
10	su1 su1/ae	6,000	172.2	0	
11	su1 su1/su2	5,500	158.8	0	
12	White waxy	1,900	15.3	3	
1	ae ae/wx	4660	25,000	151.8	0
2	ae ae/wx	4636	6,500	240.1	0
3	ae ae/wx	4612	3,150	216.6	0
4	ae ae/ae	4600	5,600	159.5	0
5	su1 su1/wx	4648	6,100	126.4	0
6	du du/wx	4624	5,700	114.4	0
7	du du/su1	4594	8,700	254.8	0
8	fl fl/0	4672	12,000	272.8	0
9	su1 su1/du	4618	10,250	233.6	0
10	su1 su1/ae	4582	2,500	53.7*	0
11	su1 su1/su2	4654	3,100	13.3	0
12	White waxy	4630	275	29.0	3
13	duduae	4588	72,00	226.5	0
14	duduwx	4666	7,300	233.4	0
15	dudusu	4642	5,100	332.9	0
16	single du	4606	1,650	99.3	0

*came out as a plug

Definitions:

Differential Scanning Calorimetry (DSC)

IR denotes initial rise

HP denotes heat peak

HF denotes heat final

CP denotes cooling peak

CF denotes cooling final.

Brookfield Viscomter

The Brookfield Viscometer measures shear-strength (in centipoise, cP) and stability of starch pastes.

Brabender Visco-amylograph Data

Pasting temperature denotes the temperature of paste formation.

Peak Viscosity denotes the temperature needed to provide a useable paste.

Viscosity at 95 C denotes the ease of cooking of the starch.

Viscosity at 50 C denotes the setback in paste viscosity during cooling of a hot paste.

Viscosity after 1 hour at 5° C. denotes the stability of the cooked paste.

Corn Percent Protein, Starch, Oil and Moisture

Percentages of oil starch and protein in corn give a measure of starch yield how recoverable the starch is.

Starch Percent Protein, Starch, Oil and Moisture

Percentages of oil starch and protein in starch give a measure of how well purified the starch is and indicates millability.

Percent Amylose and L-max

These data provide a measure of apparent amylose levels in starch.

Starch Particle Size Data

Starch particle size gives an indication of starch yield and recoverability through the milling process.

Short Hand

aeawx in table 2 refers to aeaeAE/wxWxWx, likewise duduwx=duduDU/wxWxWx Throughout this table the wild type is not listed.

FIG. 1 is a graph of enzyme activities for individual gene-dosages (e.g., MMM, mMM, mmM, mmm) of mutant alleles of the single mutants of amylose extender and dull. These data show the enzyme activities of sucrose synthase (SS), UDP-glucose pyrophosphorylase (UDPG-PP), glucokinase (GK), fructokinase (FK), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), ATP-dependent phosphofructokinase (PFK), PPI dependent phosphofructokinase (PFP), ADP-glucose pyrophosphorylase (ADPG-PP), soluble starch synthase (SSS), branching enzyme (BE) and bound starch synthase (BSS). Enzyme activities are presented as percentages relative to the wild-type control (MMM). In the case of the full mutants (mmm) there is a dramatic effect on expression levels of various enzymes in the pathway of starch synthesis. In the case of the partial mutants (mMM and mmM) there is almost no detectable change in expression levels. These data indicated that the alteration in starch quality observed with the single mutants is a consequence of the overexpression of several enzymes as well as elimination of the enzyme coded for by the mutated allele. By combining two mutant doses (e.g., wxwxWx) with other doses of another mutation (e.g., AeAeae) there would be partial reduction in two enzymes without the overexpression seen in the rest of the pathway.

FIG. 2 is a graph of the DSC scan of starches extracted from grain taken from waxy, amylose extender and common (wild type) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the high amylose starch is different from the common starch and waxy starch.

FIG. 2b is a graph of the DSC scan of starches extracted from grain taken from the double mutant (aeaeae/wxwxwx) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the double mutants is different from the

data provided in FIG. 2a on common starch and the single mutants, waxy and high amylose.

FIG. 2c is a graph of the DSC scan of starches extracted from grain taken from intermutant (aeaeAe/WxWxwx) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the intermutant starch is different from the starch of the double mutant and appears to be similar to that of waxy starch.

FIG. 2d is a graph of the DSC scan of starches extracted from grain taken from intermutant (wxwxWx/AeAeae) corn. Such DSC scans enable one skilled in the art to provide numerical data (see tables in text for data on Peak Temperature, Delta H, Peak II Temperature, Onset Temperature and Endset Temperature). It is particularly noteworthy that the profile of the intermutant starch is different from the starch of the double mutant and appears to be similar to that of waxy starch.

FIG. 3a is a graph of Brabender data taken from common starch in either neutral or acid conditions. Common corn starch shows substantial breakdown in viscosity using acid conditions.

FIG. 3b is a graph of Brabender data taken from waxy starch in either neutral or acid conditions. The waxy mutation most particularly affects viscosity of the starch in neutral conditions.

FIG. 3c is a graph of Brabender data taken from amylose extender (70% amylose) starch in either neutral or acid conditions. High amylose starches increase in viscosity in either acid or neutral conditions.

FIG. 3d is a graph of Brabender data taken from double mutant (aeaeae/wxwxwx) starch in neutral conditions. Double mutant starches maintain viscosity despite being homozygous for the waxy mutation.

FIG. 3e is a graph of Brabender data taken from intermutant (aeaeAe/WxWxwx) starch in neutral conditions. It is particularly noteworthy from these data that the new intermutant starches provide an increasing strength of viscosity similar to that seen with high amylose mutants, despite containing no increase in apparent amylose content.

FIG. 3f is a graph of Brabender data taken from intermutant (wxwxWx/AeAeae) starch in neutral conditions. It is particularly noteworthy from these data that the new intermutant starches provide an increasing strength of viscosity similar to that seen with high amylose mutants, despite containing no increase in apparent amylose content.

EXAMPLE 2

This example illustrates the production of maize grain possessing starch of the present invention. Maize plants of various backgrounds can be converted to mutant genotypes using either traditional breeding and/or backcrossing techniques or else using mutagenesis such as chemical treatments of pollen. In the alternative, waxy inbreds and hybrids can also be purchased from a number of suppliers and foundation seed companies. Any maize line with good agronomic traits and relatively high yield can be employed. In the present invention, normal inbred lines are converted to mutant inbred lines using chemical mutagenesis followed by careful selection of the mutant grain type from the segregating offspring. This method is well known to those skilled in the art (see for example, Neuffer, M. G. and Chang, M. T. 1989. Induced mutations in biological and agronomic research. *Vortr. Pflanzenzuchtg.* 16, 165-178). Any commercially valuable inbred line may be used for this process. The

lines were confirmed to carry the mutation of interest by an allelism test in which the line may be crossed with a known mutant line, a process well known to those skilled in the art. Furthermore, the kernels from the line will have the appearance and iodine-staining characteristics typical of the mutation selected, a method well known to those skilled in the art. In order to obtain the highest yields from the plants it is best next to produce a hybrid cross between two inbreds, both inbreds carrying the same mutation (e.g., both inbreds being waxy or amylose extender types). It is preferred to produce two hybrids, one being the male and being homozygous for one mutation and the other being the female and being homozygous for the other mutation. The male and female hybrids can be made up of the same or different genetic backgrounds, it is merely important for the two lines to have similar maturities in the field (i.e., require similar heat units from germination to silking and pollen shed). In order to make the intermutant cross in the field it is necessary to eliminate pollen production from the female. This can be done by a variety of methods including, but not limited to, hand pollination, hand and mechanical detasseling, introgressing genetic or cytoplasmic male sterility into the female plants, introducing male sterility through genetic transformation and use of chemical detasseling agents. The grain of this cross contains the present invention with a genotype in the endosperm of aaA/BBb, with the starch from this genotype called intermutant starch. It is well known to those skilled in the art that the genetic background can be optimized for best starch qualities.

EXAMPLE 3

Starch may be extracted from grain by a number of different methods. The most commonly used method involves a "wet milling" procedure known and used throughout the world. The basic principle involves steeping and starch separation. The key step in this process involves softening the grain in a steep tank a process which has been optimized to permit optimal separation of the corn grain components. This method was employed to extract the starch, wxwxWx/AeAeae from the grain of an intermutant developed in accordance with Example 2. The germ was easily liberated intact and freed from adhering endosperm and hull. The endosperm is macerated under water, the starch was easily separated as a white floc and gluten proteins are obtained as a yellow floc. The grain was steeped for 30-40 hrs at 48-52° C. in tanks usually holding 50-90 metric tons of grain. The steep water contains 0.2% sulphur dioxide (SO₂ gas is bubbled-in) and so is mildly acidic (pH 4.0). The sulphur dioxide helps break-up the protein matrix permitting the endosperm matrix to break-up into granules. After steeping, the grain were coarsely ground or pulped. The oil-rich embryo floats to the surface and dense starchy endosperm sinks. Separation is achieved through hydroclones (continuous separation). The starch was purified further after being milled through "an impact mill known as Entoleter mill" which smashes the slurry at high speed through counter-rotating grooved plates made of hardened steel alloy, followed by impact with an outer impact ring. The defibred starch was separated from gluten by centrifugation to give two fractions: protein (70% protein) and starch (2% protein). Processing temperature was maintained above 45° C. to prevent microbial growth. The starch was dried by flash-drying by injection into an air stream heated to 200-260° F.

Various intermutant grains can have the starch purified and prepared in this manner is suitable for a variety of food, feed and industrial uses. It may be used directly as unmodi-

fied corn starch. It may be modified by chemical or physical treatments that preserve granule structure and granules may be washed to remove residual reacts. Bleaching is sometimes used to create super-white starches. The starch can be gelatinized using a high temperature treatment and sold directly as gelatinized starch. Such starch may be chemically modified and dried. The polymer itself may be hydrolysed partially or completely to produce maltodextrins or glucose. Such products can be further modified by fermentation to produce ethanol for the gasoline industry, or the glucose can be converted to high-fructose corn syrup for the sweetener industry.

EXAMPLE 4

The mutations called shrunken-2 (sh2), brittle-2 (bt2), dull (du), sugary (su), waxy (wx) and amylose extender (ae) encode isoforms of ADP glucose pyrophosphorylase, debranching enzyme, soluble starch synthase, bound starch synthase and branching enzymes:

Shrunken-2 encodes one subunit of ADP glucose pyrophosphorylase,

Brittle-2 encodes one subunit of ADP glucose pyrophosphorylase,

Waxy encodes granule bound starch synthase,

Amylose Extender encodes an isoform of branching enzyme,

Dull alters expression of an isoforms of soluble starch synthase and branching enzyme,

Sugary alters expression and activities of soluble starch synthases and debranching enzyme.

Using known mutants and the gene-dosage crossing regimes we have examined the effects of altered gene expression on starch deposition in grain (See figures). With the bt2 mutant we see a progressive loss in measurable ADP Glc pyrophosphorylase activity which correlates well with a loss in starch synthesis in the grain. The control strength exerted by this enzyme over flux to starch cannot be quantified from these data. In fact our studies indicate that this enzyme is one of the major determinants of the duration of starch synthesis and may have little control over rate of starch synthesis. This mutation does not appreciably alter starch structure. When the mutations are with sugary, dull, waxy and amylose extender we now do detect changes in starch fine structure (branched chain length changes as well as changes in amylose/amylopectin ratios). In these cases there is more minor control of flux to starch (except with the sugary mutant which is used to make sweet-com genotypes). In all of these cases it is the changes in ratios of the starch synthases and branching enzymes which have resulted in alterations in starch fine structure. A dramatic new finding in these studies was the discovery that not only does the mutation reduce expression of key enzymes, but also it induces an overexpression of other enzymes in the pathway. Furthermore, it is only in the full mutant (mmm) genotypes where we see changes in starch fine structure demonstrating that the structural changes occur only when there is an enzyme isoform loss in combination with an enzyme isoform overexpression. Whilst not wishing to be bound by this proposal, these data illustrate the means by which starch structure may be influenced by not only reducing expression (eg using antisense constructs) an enzyme but also be simultaneously increasing expression (eg using sense construct).

Plant transformation vectors for use in the method of the invention may be constructed using standard techniques. Since these enzymes are localized in the amyloplast com-

partment of the cell, the gene construct requires the presence of an amyloplast transit peptide to ensure its correct localization in the amyloplast. The transformation construct may carry the gene either in the partial sense orientation or in the antisense orientation. Expression of said gene in the plant results in a reduction in expression of the enzyme by effects well known in the art as "sense cosuppression or antisense". When only a reduction in expression is needed the transit peptide is not required. However, when enzyme overexpression is required then a correct plastid targeting sequence is needed in the construct. Key enzymes required for this invention include branching enzyme and soluble and bound starch synthase. Branching enzyme [1,4- α -D-glucan: 1,4- α -D-glucan 6- α -D-(1,4- α -D-glucano) transferase] converts amylose to amylopectin, (a segment of a 1,4- α -D-glucan chain is transferred to a primary hydroxyl group in a similar glucan chain) sometimes called Q-enzyme. Soluble starch synthase [ADP glucose: 1,4- α -D-glucan- α -D-glucosyltransferase] extends the chain-length of amylopectin and perhaps also amylose. Bound starch synthase [ADP glucose: 1,4- α -D-glucan 4- α -D-glucosyltransferase] extends the chain length of amylose and perhaps also amylopectin.

For any antisense or sense-cosuppression construct only a partial cDNA clone is required to be expressed in a transgenic plant. Where enzyme overexpression is required, then a full length cDNA clone is needed. The sequence of maize branching enzyme-I was investigated by Baba, T., Nishihara, M., Mizuno, K., Kawasaki, T., Shimada, H., Kobayashi, E., Ohnishi, S., Tanaka, K., and Arai, Y. (Identification, cDNA Cloning, and Gene Expression of Soluble Starch Synthase in Rice (*Oryza-sativa* L) Immature Seeds. *Plant Physiology*. 103:565-573, 1993). Starch branching enzyme-II from maize endosperm was investigated by Fisher, D. K., Boyer, C. D., and Hannah, L. C. (Starch Branching Enzyme-II from Maize Endosperm. *Plant Physiology*. 102:1045-1046, 1993). The article by Mu, C., Ham, C., Ko, Y., Singletary, G. W., Keeling, P. L and Wasserman, B. P. shows an association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. *Plant Journal* 6, 151-159 (1994). The maize waxy locus for UDP-glucose starch glycosyl transferase was cloned in 1986 by Kloesgen, R. B., Gierl, A., Schwarz-Sommer, Z. and Saedler, H (Molecular analysis of the waxy locus of *Zea*. *Mol. Gen. Genet.* 203,237-244). Recently, the sequence for the maize sugary locus was observed by James, M. and Wright, A. (*The Plant Journal*) using transposon mutagenesis to locate the gene. The gene for any such protein is thought to be a debranching enzyme and may be used in constructs according to this invention.

It is believed that the chloroplast transit peptides have similar sequences (Heijne et al describe a database of chloroplast transit peptides in 1991, *Plant Mol Biol Reporter*, 9(2): 104-126). Other potential transit peptides are those of ADPG pyrophosphorylase (1991, *Plant Mol Biol Reporter*, 9:104-126), small subunit RUBISCO, acetolactate synthase, glyceraldehyde-3P-dehydrogenase and nitrite reductase. For example, the consensus sequence of the transit peptide of small subunit RUBISCO from many genotypes has the sequence:

MASSMLSSAAVATRTNPAQASM VAPFTGLKSAAF-
PVSRRKQNLDTISIAS NGGRVQC

The corn small subunit transit peptide of RUBISCO has the sequence:

MAPNVMMASSATATRTNPAQAS AVAPFQGLK-
STASLPVARRSSRSLGN VASNGGRIRC.

The transit peptide of leaf starch synthase from corn has the sequence:

MAALATSQLVATRAGLGVPDAS TFRRGAAQGLR-
GARASAAADTL SMRTASARAAPRHQQQAR-
RGGR FPSLVVC.

EXAMPLE 5

Production of fertile transgenic maize plants has been done since 1990. Although a number of DNA delivery systems are known, the selection is a particle bombardment. As noted above, constructs of the various maize mutant genes are available from depositories in the U.S. and Europe. Attached are a few examples of some of these constructs as shown in FIGS. 4a-d. FIG. 4c shows a promoter, which is CaMv (cauliflower mosaic virus), an Adh1, the waxy gene, the nos(nopoline), and the pat gene which is useful as a selectable marker and amp. FIG. 4d is similar but shows the soluble starch synthases first isoform gene in the construct. FIG. 4a again has the same construct but shows the branching enzyme first isoform. FIG. 4b shows the second branching enzyme second isoform. Of course, other constructs associated with the gene mutants used in maize breeding are also available.

For purposes of this example reference is made to FIG. 4c, the waxy construct. The purpose of this experiment is to form an inbred that has partial down regulation of the waxy gene. If the inbred selected is already a mutant for ae, then the grain produced by crossing with a non-mutant inbred will be the grain of an intermutant. Depending on the strength of the down regulation, the female inbreds grain will resemble the mm*/mm* or the mm*/m** type of starch and grain. Clearly, the transformation allows a more precise way of down regulation of the starch synthesis activity such that the alteration of the starch can be finely tuned.

To assure reasonable levels of down regulation of the waxy gene, the transformation target tissue is immature zygotic embryos, through embryogenic callus can also be employed. Immature zygotic embryos from A188 plants 12 days after pollinated with the B73 ae inbred can be selected. The medium for the callus was 6 mM L-proline, 2% (w/v) sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3% (w/v) Gelrite (Caroline Biological Supply) (pH 6.0). Callus is grown and suspension cultures were initiated.

A MS-based liquid medium containing 100 mg/l myo-inositol, 2 mg/l 2,4-D, 2 mg/l 1-naphthalenacetic acid (NAA), 6 mM proline, 200 mg/l casein hydrolysate (Difco Laboratories), 3% (w/v) sucrose, and 5% (v/v) coconut water (Difco Laboratories) (pH 6.0). Cell suspensions were maintained in this medium in 125 ml Erlenmeyer flasks at 28° C. in the dark on a gyrating shaker at 125 rpm.

The transformation vector in FIG. 4c is selected. This plasmid contains a 355-ladh-pat nos 3' selectable gene expression cassette.

The cell suspensions are sieved and then suspended in 5 ml of suspension medium and placed on filter paper through vacuum. The construct was coated into particles as is known in the art. The plates were then bombarded. The cells are then transferred to a N-6 medium and after 14 days transformed cells are selected by 1 mg/l bialaphos. The cells are then suspended in a medium containing 0.6% (w/v) (Sea-Plaque; FMC) and held at 37° C.

Two to five weeks later, growing calli are removed and transferred to the surface of fresh selection medium. Plants were regenerated in a MS based medium having 6% sucrose 1 g/l myo-inositol, 1 mg/l NAA (34), and 0.3% (w/v) Gelrite (pH 6.0). Next the embryo germination occurred in a MS

media of 0.25 mg/l NAA and 3% (w/v) sucrose and light. Plants are grown and transferred to the greenhouse. The expression levels in the plant can then be evaluated.

The plant is bred and developed to an inbred having the mutant and the down regulated pathway. Alternatively, the selected inbred can have the mutant crossed onto a transgenic after transformation to form the desired starch in the grain when the transgenic plant is employed as the female.

EXAMPLE 6

This example illustrates the gelatinization temperature of the starch of present invention compared to other starches. The gelatinization temperatures are listed in Table 3 below.

TABLE 3

Starch Samples	% Amylose*	Gelatinization* Temperature ° C.
1 Native common maize	28	71
2 AMY V native	57	80
3 AMY VII native	73	90
4 Present Invention	21	73
5 Native <u>ae</u> wx	25	80
6 Native wxwxwx	3	72

*Values rounded to a whole number

Sample 1 was a commercial product sold by American Maize-Products Company of Hammond, Ind. The percent amylose and the gelatinization temperature for Sample 1 above are mean values determined by random sampling of product. The 99% confidence level for percent amylose and gelatinization temperature are 25.9 to 29.3 and 68.7 to 72.9, respectively.

AMY V and AMY VII are commercial high amylose corn starches sold by American Maize-Products Company of Hammond, Ind. The percent amylose and the gelatinization temperatures in Table 3 above are mean values determined from a random sampling of product. The 99% confidence interval for the percent amylose in AMY V and AMY VII was 53.4 to 62.5 and 65.5 to 73.8, respectively. The 99% confidence interval for the gelatinization temperature for AMY V and AMY VII was 72.8 to 84A and 83.1 to 90.8, respectively. Both AMY V and AMY VII were grown in native maize.

Starch Sample 4 corresponds to the Present Invention, while Sample 5 corresponds to the average values from Example 1 of U.S. Pat. No. 5,009,911. Sample 6 corresponds to a commercial waxy starch sold by American Maize-Products Company.

The method for determining both the percent amylose and the gelatinization temperature was:

The percent amylose was determined using standard calorimetric iodine procedures wherein the starch is first gelatinized with sodium hydroxide and then reacted with an iodine solution and the resulting sample measured using a spectrophotometer in a 1 cm cell at 600 nm against a blank of 2% iodine solution.

The DSC gelatinization temperature was measured using a scanning calorimeter manufactured by Mettler Moddle No. 300 using 30% starch solids following the procedure outlined in the owner's manual for that model.

It is readily apparent from Table 3 above that the gelatinization temperature of the starch of the present invention is comparable to common corn starch.

EXAMPLE 7

This example illustrates the gel strength of a sol made from the corn starch of the present invention compared to a

sol made from aewx corn starch, a sol made from common corn starch, and a sol made from wxwxwx starch. The results of the test are reported in Table 4 below.

TABLE 4

Sample	Strength (grams)
Present Invention	159.5
Common Corn Starch	225.0
aewx Corn Starch	55.0
Waxy Corn Starch	16.0

In order to perform the gel strength test reported in Table 4 above, sols were prepared by mixing water with starch and subjecting the slurry to a rapid heat mode in the Brabender Visco-Amylograph to heat the sample to 50° C. Once 50° C. was reached, the instrument was set at a controlled rate of heating, 1.5° C./minute, until a temperature of 95° C. was reached. The sample was then held at 95° C. for 30 minutes. Next, the sample was cooled at 1.5° C. to a temperature of 50° C. for 30 minutes. Portions of these sols were added separately to 4 ounce jars into which a plunger was placed. The sols were then allowed to stand at ambient conditions for 24 hours. Gel strength was measured by determining the force needed to remove the plunger from the sol.

This example illustrates that the gel strength of a sol made in accordance with the present invention is comparable to common corn starch sols.

EXAMPLE 8

This example illustrates the difference between aewx starch, a waxy starch wherein the plant had a triple dose of the waxy gene, and the starch of the present invention. All starches were obtained from maize.

All starches were tested for their rheological properties. Each starch was subjected to the same test procedure using the same method. The starch granules were pasting using a Brabender Visco-Amylograph with the cooling probe down and with the 750 g cm cartridge. The starch slurry, 5.5% initial solids, was rapidly heated to 60° C. in the Brabender cup, and then pasted while increasing the temperature to 95° C. at 1.5° C. per minute. The starch paste was held at this temperature for 20 minutes, and then immediately loaded onto the measuring geometry of the rheometer which had been preheated to 70° C. A four-part rheological characterization was performed. The gel cure segment which monitored the formation of structure at 0.2 hertz and 0.2% strain (well within the linear viscoelastic region) was measured as the sample was cooled from 70° C. to 25° C. and held for 4 hours. Also measured was the strain sweep, which measures the Rheological response of the paste or gel to increasing levels of strain, also at 0.2 hertz, at 25° C.

Using this technique, distinct differences between aewx starch and the starch of the present invention were found. FIG. 5 shows the results of the gel cure analysis. The starch of the present invention, while having an initial Modulus (G') lower than aewx starch, more quickly formed structure or gelled as evidenced by the rapid rise in G'. Thus the starch of the Present Invention was distinct from aewx starch in the rate of gel formation despite similar apparent iodine binding contents. FIG. 6 shows the results of the strain sweep analysis only for the starch of the present invention and the aewx starch. Here the starch of the present invention showed dilatant behavior as evidenced by the increase in G' as the strain level was increased. Under the applied levels of strain, the structure was not destroyed. In contrast, the structure of

the aewx starch was rapidly destroyed when the applied strain became greater than 4%. Thus, when compared to aewx starch, the starch of the present invention formed a gel which didn't break under the applied strain of this test. In contrast, aewx starch showed much less time-dependent structure formation and did "break" or was destroyed by the strains applied in this technique.

EXAMPLE 9

This example illustrates preparing a thickener composition in accordance with the present invention.

The starch of the present invention is mixed with water in an amount to produce a slurry having 10% by weight starch. The sol has a short texture and a bland taste. The sol when cooked at about 90° C. for 10 minutes produces a thickener composition which had better clarity than a similar thickener composition made from common corn starch and a shorter texture.

EXAMPLE 10

This example compares the mouth feel of a gel made from the starch of the present invention to a gel made with a common starch.

The common starch and starch of the present invention were pasted using a Brabender visco-amylograph. The starch was slurried at 5.5% solids, and then heated using the rapid heat mode to 50° C. Using controlled heat of 1.5° C. per minute the slurries were heated to 95° C., and then held at this temperature for 30 minutes. The final solids was 5.9%. The sample starch pastes were then poured into small jelly jars, covered with cellophane, and allowed to age 24 hours before analysis. A taste panel was then asked to rank the samples for the following attributes.

First they ranked the two for relative firmness to the touch.

Common	Present Invention
5.1	5.6

Next, they ranked the relative break, firmness, and clearing of this sample while being masticated.

	Common	Present Invention
Degree of clean break	3.2	8.5
Firmness	4.4	4.5
Rate of clearing	2.5	4.6

These results show that the firmness of these samples is similar. However the starch of the present invention has a much cleaner break while being masticated.

Furthermore, it tends to clear from the mouth faster than a common based starch gel. The panel all agreed that the starch of the present invention produced a gel which had a "clean" mouth feel similar to that of a gelatin or a pectin.

EXAMPLE 11

This example illustrates making a gum candy using the starch of the present invention.

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The following ingredients and procedure are used:

TABLE 5

Ingredients	% by Weight Present Invention
44/62 Corn Syrup Unmixed	56.34
Sugar, fine granular	23.98
Water	7.73
Present Invention Starch	11.80
Citric Acid	0.07
Sodium Citrate	0.08
	100.00

Procedure

All ingredients are mixed and then cooked to 340° F. using conventional equipment such as a jet cooker. The cooked slurry is then poured into candy molds and allowed to solidify.

EXAMPLE 12

This example illustrates making a Bavarian cream pie using starch of the present invention.

The following ingredients and procedure are used:

TABLE 6

Ingredients	% by Weight Present Invention
Whole milk, fresh 3.5%	72.794
Sugar, fine grain	17.586
Salt, Flour	0.101
Present Invention	5.410
Banana Flavoring	0.300
Egg Yolk, fresh	3.809
	100.000

Procedure

All of the pie filling ingredients except for egg yolks are combined and cooked at 195° F. for 3 to 5 minutes. Then the ingredients are cooled to 120° F. with constant stirring. Next, egg yolks are added and the admixture well blended. This mixture is then added to a conventional pie crust and allowed to cool to room temperature before serving.

EXAMPLE 13

This example illustrates preparing a lemon pie filling with the starch of the present invention.

The following ingredients and procedure are used:

TABLE 7

Ingredients	% By Weight Present Invention
Water	62.94
Sugar	19.30
Maltodextrin	6.67
Present Invention	4.50
Corn Syrup Solids	2.50
Lemon Juice	2.50
Vegetable Shortening	1.03
Salt	0.23
Citric Acid	0.20
Emulsifier	0.10
Lemon Oil (2x)	0.03
	100.00

Procedure

Half of the water is combined with the sugar and brought to a boil. All of the remaining ingredients are slurried

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together and then added to the boiling sugar and water. The temperature of this mixture is then adjusted to 200° F. and held there for two minutes. The mixture is then poured into prepared pie crusts and allowed to cool and solidify.

EXAMPLE 14

This example illustrates making a chocolate mousse using the starch of the present invention. The formulation in Table 8 is employed to prepare a mousse mix.

TABLE 8

	%
Frodex 24-924	39.20
Sugar (Baker's)	30.75
Whiptreme 3554 (Kerry Ingredients)	12.88
Starch of Present Invention	9.80
Cocoa, Dutch Red (Gill & Duffus Products)	7.17
Leceitreme 40 (Kerry Ingredients)	0.20
Flavor	as desired
	100.00

Procedure

Combine ingredients to form a uniform blend.

Use

Combine 200 gms. of mousse mix with 1 cup (250 grs.) milk. Using an electric mixer, combine on low speed for 1 minute. Scrape bowl. Mix on high speed for 3 minutes, until light and fluffy. Spoon into serving dishes and refrigerate for 1 hour before serving.

To prepare the mousse mix, all the ingredients are mixed. To prepare the mousse itself, 200 grams of mousse mix are combined with 250 grams of milk and combined at a low speed. Then the mixture is stirred at a high speed to make it light and fluffy and the mixture is refrigerated for one hour. In this way, a light, fluffy mousse is prepared

Accordingly, the present invention has been described with some degree of particularity directed to the preferred embodiment of the present invention. It should be appreciated, though, that the present invention is defined by the following claims construed in light of the prior art so that modifications or changes may be made to the preferred embodiment of the present invention without departing from the inventive concepts contained herein.

We claim:

1. A plant having, a wild-type triploid endosperm genotype composed of 1 or 2 doses of a recessive mutant allele of a first gene and 1 or 2 doses of a recessive mutant allele of a second gene wherein said first and second genes are independently selected from the group of genes consisting of waxy, amylose extender, dull, horny, sugary, shrunken, brittle, floury and opaque; such that when said first gene and said second gene are not waxy, said wild-type triploid endosperm genotype is not homozygous recessive at the waxy gene.

2. The plant of claim 1 wherein said plant is a corn plant.

3. A plant according to claim 1, wherein said first and second genes are independently selected from the group consisting of amylose extender, dull, horny, sugary, brittle, floury and opaque.

4. A plant according to claim 1, wherein said first and second genes are independently selected from the group consisting of waxy, dull, horny, sugary, shrunken, brittle, floury and opaque.

5. A plant having a wild-type triploid endosperm genotype composed of 1 or 2 doses of a recessive mutant allele of a first gene and 1 or 2 doses of a recessive mutant allele of a

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second gene and 1 or 2 doses of a recessive mutant allele of a third gene wherein said first and second and third genes are independently selected from the group of genes consisting of waxy, amylose extender, dull, horny, sugary, shrunken, brittle, floury and opaque; such that when said first gene, said second gene and said third gene are not waxy, said wild-type triploid endosperm genotype is not homozygous recessive at the waxy gene.

6. The plant of claim 5 wherein said plant is a corn plant.

7. A plant according to claim 5, wherein said first, second and third genes are independently selected from the group consisting of amylose extender, dull, horny, sugary, brittle, floury and opaque.

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8. A plant according to claim 5, wherein said first, second and third genes are independently selected from the group consisting of waxy, dull, horny, sugary, shrunken, brittle, floury and opaque.

9. A grain produced from, or a seed that produces, a plant of any one of claims 1-8.

10. A plant according to any one of claims 1-8 wherein said plant is a starch producing plant.

11. A plant according to claim 10 wherein said plant is a corn plant.

* * * * *